NR2F1 contributes to cancer cell dormancy, invasion and metastasis of salivary adenoid cystic carcinoma by activating CXCL12/CXCR4 pathway

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Abstract

Background: Salivary adenoid cystic carcinoma (SACC) can recur after removal of the primary tumor and treatment, where they can keep no clinical symptoms and dormant state for 10–15 years. NR2F1 has been demonstrated to regulate the tumor cell dormancy in various malignant tumors and has a potential impact on recurrence and metastasis of carcinoma. However, the role and significance of NR2F1 in SACC dormancy still remain unknown.

Methods: A total number of 59 patients with a diagnosis of SACC were included to detected expression of NR2F1, Ki-67 by immunohistochemical (IHC) staining and terminal deoxynucleotidyl transferase-mediated dUTP nick and labeling (TUNEL). Fisher’s exact test was used to examine the NR2F1 expression and clinicopathologic parameters of SACC. In vitro, SACC cell lines were transfected NR2F1 and knockdown NR2F1 respectively. CCK-8, flow cytometry, wound healing assay and transwell invasion determined SACC cell proliferation, apoptosis, cell cycle, migration and invasion respectively. Chromatin immunoprecipitation (ChIP) assays were utilized to demonstrate the potential role of NR2F1 in SACC invasion via CXCL12/CXCR4 axis. In vivo, xenografts of nude mice via subcutaneous injection or tail vein injection were used to testify the results in vitro.

Results: Among the 59 patients with SACC, 23.73% (14/59) were positive to NR2F1 expression, a lower rate of expression compared with 60% (6/10) in normal salivary gland samples. NR2F1 was correlated with metastasis, relapse and dormancy of SACC. SACC cells with transfected NR2F1 remained dormant, as well as enhanced invasion and metastasis. Knockdown of NR2F1 via siRNA after NR2F1 overexpression restored the proliferation and the cell number in G2/M phases, and reduced the abilities of migration and invasion. In addition, NR2F1 promoted the expression of CXCL12 and CXCR4, and overexpression of CXCL12 at least partly rescued the proliferation, migration, and invasion activities induced by NR2F1 silencing.

Conclusions: NR2F1 may be an underlying mechanism of SACC recurrence and metastasis via regulating tumor cell dormancy through CXCL12/CXCR4 pathway.

Keywords: Salivary adenoid cystic carcinoma (SACC), nuclear receptor subfamily 2 group F member 1 (NR2F1), tumor dormancy, tumor invasion, metastasis

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Background
Salivary adenoid cystic carcinoma (SACC) is one of the most common malignant salivary gland tumors, accounting for about 28% [1, 2]. Five-year survival rates for patients with SACC are 50–90% but drop to 50% after 10 years, and 20% after 20 years. SACC patients usually suffered from metastatic relapse several or decades years after they had undergone radical surgery [3, 4]. This phenomenon has become a puzzle for a long time till cancer dormancy was raised, which will have potential to explain this prevalent clinical behavior of SACC patients [5].

Cancer dormancy, mentioned in 1864 [6] and described in 1959 [7], has been historically defined in clinical terms to describe the hypothetical state of cancer cells lying in wait over a period of time after treatment of the primary tumor, pending subsequent growth and clinical recurrence [8]. The mitotic arrest actually got a real sense of dormancy, which precisely referred to cellular dormancy, suggesting that a G0/G1 arrest can exist in certain cancer cells [9, 10]. Angiogenic dysfunction and immunologic regulation are responsible for tumor mass dormancy with a sound-equilibrium between dead cells and proliferative cells [11–14]. In accordance with the properties of tumor dormancy including insensitivity to radiotherapy and chemotherapy, and escapable from immune-surveillance [15, 16], it deems to be the “seeds” for tumor relapse and metastasis.

Recent studies have shed significant light on the molecular mechanisms governing the invasion and dissemination phase of metastasis through cancer dormancy. Kim et al. demonstrated that suppression of two dormancy genes, BHLHE41 and NR2F1, increased the growth of ER positive MCF7 cells in vivo [17]. And disseminated ER positive tumor cells carrying a dormancy signature were more likely to undergo prolonged dormancy before resuming metastatic growth [17]. Using computational tools, Adam et al. found that p38 transcriptionally regulated a core network of 46 genes that included 16 TFs in head and neck squamous cell carcinoma (HNSCC), which played key roles in tumor suppression and induction of tumor cell dormancy [18]. Bragado et al. showed that TGF-β2 and TGF-β-RIII signaling through p38α/β regulated the dormancy of disseminated tumor cells (DTCs) and defined restrictive (BM) and permissive (lung) microenvironments for HNSCC metastasis [19]. However, in spite of these significant advances, the mechanism of cancer dormancy elucidating the post-dissemination phase of metastasis has remained less understood.

NR2F1 (nuclear Receptor subfamily 2 group F member 1, or COUP-TF1) is one of NR2F family and modulates gene expression during cancer development and growth [20]. Recently, NR2F1 has been shown to be associated with cancer cell dormancy in HNSCC [21]. Here, we evaluated the correlations between NR2F1 expression and tumor cell dormancy, and the clinical pathological characteristics of SACC patients. SACC cells with NR2F1 over-expression and NR2F1 knockdown were used to investigate the differences of biological behaviors including proliferation, cell cycle, apoptosis, migration and invasion. Finally, the mechanism of NR2F1 contributing to cancer cell dormancy, invasion and metastasis of SACC cells was investigated. Our findings showed that in NR2F1 over-expressed tumor cells, proliferation and cell cycle could remain arrested, but invasive and metastatic properties could be enhanced. This observation might have important implications in the therapeutic options for SACC patients.

Methods
Tissue sample collection
The cohort was obtained from patients who were histologically diagnosed as SACC and underwent radical surgery at West China Hospital of Stomatology, Sichuan University from January, 2004 to December, 2007. Tumors were staged and graded according to the American Joint Committee on cancer. Exclusion criteria included recurrence, preoperative radiotherapy, chemotherapy or biotherapy, and incomplete medical records. Finally, 59 patients (28 males and 31 females; median age, 42 years, range from 22 to 77) were recruited in this study. Immunohistochemical analysis for the formalin-fixed, paraffin-embedded specimens from these patients. This study was approved by the Institutional Ethics Committee of the West China Medical Center, Sichuan University, China. Pathologic characteristics of the tumors and clinical data of the patients were summarized in Table 1.

Immunohistochemical staining
Anti-NR2F1 (1:200, abcam) and Ki-67 (1:400, Cell Signaling Technology) were used for Immunohistochemical staining. Negative was graded as 0 to 10% within 4–6 microscopic fields at × 400 magnification and positive was graded as more than 10% as well.

TUNEL assay
Terminal deoxynucleotidyl transferase-mediated dUTP nick and labeling (TUNEL) Kit (KeyGEN) was to determine the cell apoptosis. Negative was graded as 0 to 10% within 4–6 microscopic fields at × 400 magnification and positive was graded as more than 10% as well.

Cell culture and transfection
SACC-83 and SACC-LM cell line have been purchased from Shanghai Life Science College Cell Resource Center, Chinese Academy of Sciences and conserved in State Key Laboratory of Oral Diseases. For in vitro assays, cells were seeded at 2 × 10⁵/ml. For the NR2F1 induction experiment, SACC-83 and SACC-LM cells were grown in RPMI 1640 with 10% FBS and 1% P/S and transfected with pGS5-empty or pGS5-NR2F1.
NR2F1 transient siRNA knockdowns

SiRNAs targeting NR2F1 (NR2F1-Homo-2112 (siRNA-1), NR2F1-Homo-2838 (siRNA-2), Human NR2F1 (siRNA-3)) and control siRNA (siControl) were purchased from Genechem. The target sequence was: siRNA-1: GCCUCA AGAAGUGCCUCAATT, UUGAGGCACUUCUUGAGG TTT; siRNA-2: UCAUCGAGCAGCUCUUCUUTT, AAGA AGAGAGCUGCUCGAUGATT; siRNA-3: CUCUCAUCCGCAUAGUUTT, ACAUAUCGCGGAUGAGAGTT; siControl: UUCUCCGAACGUGUCACGUTT, ACGUGACAGUCCGAGAATT. Transient transfection in SACC cells was performed using 20 μM of each siRNA with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Knockdown was verified by real time qRT-PCR.

Immunofluorescence

SACC cells were seeded into coverslips (1 x 10^4/ml) and cultured in a 12-well culture plate for 24 h. After washed in cold PBS, the cells were fixed in 4% parafomaldehyde for 20–25 min and blocked in 1% bovine serum albumin for 30 min at room temperature. Rabbit anti-NR2F1 (abcam, 1:200) and FITC-conjugated goat anti-rabbit IgG (1:500; Zhongshan Goldenbridge) were orderly used to incubate these cells. 4’-diamidino-2-phenylindole (DAPI; 1 μg/μL) was used to determine the cell nucleus. The results were collected by a fluorescence microscope (Olympus).

Real time reverse transcriptase PCR (qRT-PCR)

One Step PrimeScript™ RT-PCR Kit (TaKaRa) was for Real time qPCR and the results were analyzed by Applied Biosystems ABI PRISM 7300. NR2F1/TF-COUP1: forward: GCCTCAAAGCCATCGTGCTG; reverse: CCTCACGTA CTCTTCCAGTG. GAPDH was used as an internal control for the normalization of target gene expression.

Western blot

Rabbit anti-NR2F1 (abcam, 1:1000) and 1:3000 dilution of anti-rabbit IgG secondary antibody (ZSGB-BIO, China, 1:1000) were to determine the protein expression. Rabbit anti-Lamin B (ZSGB-BIO, China, 1:1000) was used as an internal control. Images were acquired with a ChemiDoc Touch imager (Bio-Rad) and quantification was done using Quantity One 4.4.0 software.

Franks of the cancer were performed by Cell Counting Kit (CCK)-8 assay according to the manufacturer’s protocol (DOJINDO, Japan).

Cell cycle analysis

Cells were collected by centrifuge with disposed upper layer and then fixed and stained for total DNA with propidium iodide (PI) using Cell Cycle Detection Kit (KeyGEN). Data was acquired with a Beckman Coulter flow cytometer.

Wound healing assay

In vitro cell invasion assays were performed with QCM−96-well cell invasion assay kit (Chemicon International, Temecula, CA, USA). After 24 h, the tumor cells were stained by Crystal violet and photographed under microscopy (x 100) as previously described.

Xenografts

Balb/c immunodeficient nude female mice (Laboratory Animal Center of Sichuan University, Chengdu, China), aged 3 weeks were used. 20 mice were randomized and divided into two groups (NR2F1high, negative control), 10 mice each. Tumor cells were then injected via subcutaneous (2.5 x 10^6 cells/100 μl PBS/mouse) on the back of nude mice. Tumor growth was then monitored using caliper.
measuring the mice were euthanized with a dosage of 150-200 mg/kg Pentobarbital Sodium via intraperitoneal injection after 4 weeks and tumors were harvested after 4 weeks and fixed by 4% paraformaldehyde and then embedded by paraffin for hematoxylin-eosin (HE) staining and IHC analyses. Another 10 mice were grouped as above and tumor cells were injected via tail vein (10^5 cells/100 μl PBS/mouse). The lung tissues were excised after 4 weeks for HE staining to detect micro-metastasis.

**Chromatin immunoprecipitation (ChIP) assays**

ChIP assays were performed using a ChIP Assay Kit (Abcam) according to the manufacturer’s instructions. Briefly, cells were fixed, lysed, and sonicated to obtain DNA fragments in arranging in size from 200 to 1,000 bp. Chromatin was then precipitated with nonspecific IgG antibodies (Sigma), ChIP-grade rabbit anti-NR2F1 (Abcam), or ChIP-grade rabbit anti-H3 (Abcam). DNA was extracted and PCR was performed with primers for CXCL12, CXCR4 and CXCR7 promoter fragments.

**Statistical analysis**

All data are presented as the mean ± standard deviation of at least 3 independent experiments. Graph construction and statistical analysis were performed using SPSS 17.0 and GraphPad Prism 5.0. The correlation between NR2F1 and clinicopathologic parameters in all patients was analyzed through the Fisher’s exact test. P values were calculated to determine statistical significance of the results. *p < 0.05 and **p < 0.01 were considered statistically significant.

**Results**

**High expression of NR2F1 associates with the metastasis, relapse and dormancy of SACC patients**

To investigate the clinic significance of NR2F1 in human SACC cases, we first applied immunohistochemistry staining to detect NR2F1 expression in 59 SACC patients. The result showed that NR2F1 reactivity was generally detected in nuclei, and only occasionally in the cytoplasm. The positive expression of NR2F1 was 23.73% (14/59) in SACC and 60% (6/10) in normal salivary gland samples, respectively (Fig. 1A). There was significant difference of NR2F1 expression between SACC and normal salivary gland samples (p<0.05).

The correlation between the expression of NR2F1 and clinicopathologic parameters of SACC was presented in Table 1. NR2F1 expression was higher in cases of SACC with recurrence and metastasis than that in cases without recurrence and metastasis (p = 0.0321, p = 0.0112, respectively). However, NR2F1 expression in patients with local invasion was similar to patients without local invasion (p = 0.1488). The level of NR2F1 in stage I-II was the same as that in stage III-IV(p = 0.7592). In addition, there was no statistically significance association of the NR2F1 positive expression status with age and sex (p>0.05). These indicated that NR2F1 expression was significantly related to the recurrence and metastasis of SACC patients.

Next, we detected the proliferation and apoptosis of tumor cells in NR2F1-posive and NR2F1-negative SACC samples. In NR2F1-positive areas, the expression of Ki-67 was 0~1% and TUNEL assay was negative. In NR2F1-negative areas, the expression of Ki-67 was 3~5% and TUNEL assay was positive (Fig. 1B). These indicated that NR2F1\textsuperscript{high} cancer cells were neither proliferative nor dead and consistent with a dormant phenotype in SACC cells.

**NR2F1\textsuperscript{high} SACC cells are dormant but highly migratory and invasive**

To determine the function of NR2F1 in SACC cells in vitro, we performed NR2F1 overexpression via lentivirus transfection (Fig. 2A-C). We first investigated the influence of NR2F1 high expression on the proliferation of SACC cells using CCK-8 assays. As shown in Fig. 3A, NR2F1 high expression inhibited the proliferation of SACC-83 and SACC-LM cells, compared with the control (p < 0.05). This change in proliferative activity was confirmed by flow cytometry analysis of cell cycle, which showed that compared with the control, there were more NR2F1\textsuperscript{high} SACC cells in G0/G1 phases and less cells in G2/M phases (p < 0.05, Fig. 3B). Meantime, no significant difference of cell apoptosis was observed between NR2F1\textsuperscript{high} SACC cells and the control (p > 0.05, Fig. 3C). Then, we applied wound-healing and transwell invasion assays to investigate the effect of NR2F1\textsuperscript{high} on the migration and invasion of SACC-83 and SACC-LM cells. The data showed that NR2F1 high expression in SACC-83 and SACC-LM cells increased cancer cell migration and invasion abilities at approximately 75 and 70%, respectively, compared with control (Fig. 3D-3E). These indicated that NR2F1\textsuperscript{high} SACC cells possessed dormancy and dormant cells had higher migration and invasion abilities.

**NR2F1\textsuperscript{low} SACC cells are proliferative but low migratory and invasive**

To further verify whether the effect of NR2F1 high expression on the dormancy of SACC-83 and SACC-LM was unique, we used siRNA-NR2F1 in NR2F1\textsuperscript{high} SACC cells and observed that the down-regulation of NR2F1 not only restored the proliferation and the cell number in G2/M phases of SACC-83 and SACC-LM cells, but also decreased the migration and invasion abilities of SACC-83 and SACC-LM cells (Fig. 4A-D). And the down-regulation of NR2F1 had not obviously changed the apoptosis number of SACC-83 and SACC-LM cells (Fig. 4E). These indicated that NR2F1 silencing promoted the proliferation of SACC cells, which indicated
that down-regulation of NR2F1 promoted SACC-83 and SACC-LM to exit from dormancy.

**Overexpression of NR2F1 inhibits tumor growth and promoted invasion and metastasis in an xenograft model**

We then established an xenograft model using NR2F1 high SACC cells and SACC cells respectively and documented the tumor volume weekly within one month. As Fig. 5A was shown, NR2F1 high groups exerted a significantly slower tumor growth than the control. Furthermore, the volume of NR2F1 high tumor was smaller than that of the negative control tumor at the end of 4 weeks (Fig. 5B). These results suggested that NR2F1 high cancer cells developed overt tumor in vivo with a slow speed and presented a state of dormancy in vitro.

In agreement with in vitro data, one nude mouse (10%) implanted with SACC-83 via the tail vein produced spontaneous lung metastasis, and 100% of the mice with NR2F1 high SACC-83 had lung metastases, indicating that metastasis was promoted by high NR2F1 expression (Fig. 5C). And NR2F1 high SACC-83 cells appeared to develop metastases to lungs more quickly. HE staining confirmed that there were tumor metastatic lumps in the lung tissue of NR2F1 overexpressed group, which was confirmed by IHC (Fig. 5D-5E), while cancer cells were not found in the liver tissue. These suggested that NR2F1 high cells were prone to be more invasive and easier to metastasis than NR2F1 low cells.

**NR2F1 promotes the expression of CXCL12 and CXCR4**

Recent studies have shown CXCL12/CXCR4 pathway plays a pivotal role in invasion and metastasis of SACC cells [22, 23]. To investigate whether CXCL12 and CXCR4 in SACC cells were regulated by NR2F1, we further confirmed the expression of CXCL12 and CXCR4 in response to NR2F1 knockdown and overexpression through RT-PCR. We found that knockdown of NR2F1 down-regulated the expression of CXCL12 and CXCR4, while overexpression of NR2F1 up-regulated the expression of CXCL12 and CXCR4 in SACC-83 and SACC-LM cells (Fig. 6A). And CXCR7, another receptor of CXCL12, had not obviously changed while NR2F1 was knockdown and overexpressed. Furthermore, we found NR2F1 directly bound to CXCL12 and CXCR4 promoters, not CXCR7 promoter, as determined by ChIP assay (Fig. 6B). This suggested that NR2F1 was an essential factor for CXCL12/CXCR4 signaling.
Overexpression of CXCL12 rescues SACC cell behaviors inhibited by NR2F1 silencing

To investigate whether NR2F1 regulated SACC cell behaviors via CXCL12/CXCR4 pathway, we examined the effect of rhSDF-1a (10 ng/ml, exogenous CXCL12) on SACC cells. Introduction of exogenous CXCL12 successfully restored the CXCL12 expression suppressed by NR2F1 silencing, compared with the control. Overexpression of CXCL12 rescued the reduced migration and invasion in NR2F1-depleted SACC-83 and SACC-LM cells, at least in part, as shown in Fig. 6C, 6D. Furthermore, the enhanced proliferative activity by NR2F1 depletion was almost abrogated by CXCL12 overexpression (Fig. 6E). Taken together, these results indicated the role of NR2F1 in regulation of SACC cell behaviors was mainly mediated by CXCL12/CXCR4.

Collectively, NR2F1 may be a marker for SACC tumor cell dormancy and high expression of NR2F1 in SACC may be useful to identify patients at high risk for recurrence and metastasis.

In this study, we show that compared to the normal salivary gland, SACC samples contained smaller amounts of NR2F1, which was in accordance with NR2F1 expression in mammary tumor and HNSCC [21, 25]. However, in prostate cancer, esophageal cancer and melanoma, NR2F1 exhibited a higher expression compared with non-tumor samples [26–28]. This difference might attribute to different kinds of human carcinoma and different sample sources. We further found that the expression of NR2F1 was associated with local recurrence and metastasis according to the results from pathological section staining of SACC patients. This is in line with the present reports that NR2F1 has been demonstrated to serve as a critical marker for tumor dormancy.

**Discussion**

Tumor dormancy has been demonstrated to empower the tumor recurrence and metastasis in many types of cancers, including breast cancer, prostate cancer, melanoma and HNSCC [5, 19, 24]. In this study, we found that high expression of NR2F1 was strongly associated with recurrence, metastasis and dormancy of SACC patients. NR2F1 overexpression in SACC cells could reduce cell proliferation and arrest G0/G1 phases, as well as enhance migration and invasion activity. Mechanistically, overexpression of CXCL12 rescued the proliferation, migration, invasion activities induced by knockdown of NR2F1 in SACC cells, at least in part, indicating that the role of NR2F1 in regulation of SACC cell behaviors was mainly mediated by CXCL12/CXCR4.
regulator in angiogenesis and lymphangiogenesis to promote tumor invasion and metastasis [29–31]. Huang et al. found that the expression of IncRNA NR2F1-AS1 was up-regulated in chemo-resistant hepatocellular carcinoma and could promote the invasion, migration and drug-resistant in vitro [32]. Jiang et al. demonstrated that dietary supplements could suppress metastatic behavior of prostate cancer cells by down-regulating the expression of NR2F1 [33].

Then, we showed that both in SACC samples and SACC cell lines, NR2F1-high cancer cells displayed neither a proliferative nor an apoptotic state, namely a state of dormancy. As expected, NR2F1 silencing stimulated SACC cell growth in vitro. Intriguingly, we noticed that NR2F1-high cancer cells performed an enhanced invasion and migration in vitro and an advanced metastasis in vivo. These not only identify NR2F1 as a marker of SACC dormancy, but also a

Fig. 3 Effect of NR2F1 overexpression on the dormancy, migration and invasion of SACC-83 and SACC-LM cells. (a) CCK8 assay was used to examine the cell growth rates in control and NR2F1-high SACC cells group. The data showed that the cell growth rates were significantly suppressed in NR2F1-high SACC cells. Error bars represent the mean ± SD of triplicate experiments. *p < 0.05. (b) Flow cytometry was used to examine the cell cycle in control and NR2F1-high SACC cells group. Representative figures of three independent experiments were shown. Compared with the control, there were more NR2F1-high SACC cells in G0/G1 phases and less cells in G2/M phases (p < 0.05). (c) Flow cytometry showed cell apoptosis in control and NR2F1-high SACC cells group. Apoptotic analysis of SACC cells showed no difference between control and NR2F1-high SACC cells group (p > 0.05). Representative figures of three independent experiments were shown. (d) Migration assay examined the cell migration ability in control and NR2F1-high SACC cells group. Representative figures of three independent experiments were shown. NR2F1 high expression could promote the migration ability of SACC cells. The mean was derived from cell counts of 3 fields, and each experiment was repeated 3 times. Error bars represent the mean ± SD of triplicate experiments. *p < 0.05. (e) Invasion assay examined the cell invasive ability in control and NR2F1-high SACC cells group. Representative figures of three independent experiments were shown. NR2F1 high expression could promote the invasion ability of SACC cells. The mean was derived from cell counts of 3 fields, and each experiment was repeated 3 times. Error bars represent the mean ± SD of triplicate experiments. *p < 0.05.
mediator for the process of tumor metastasis. Many studies have identified NR2F1 as a marker of tumor cell dormancy in breast cancer, HNSCC, prostate cancer, etc. In breast cancer, Borgen et al. [34, 35] analyzed the NR2F1 expression in DTCs by double immunofluorescence (DIF) staining of extra cytospins prepared from 114 BM samples from 86 selected DTC-positive breast cancer patients, and found NR2F1 as a marker of dormancy in breast cancer. Cackowski et al. [36] demonstrated that MERTK, one of TAM family of receptor tyrosine kinases, being knockdown could induce a G0/G1 arrest in prostate cancer cells via increasing expression of NR2F1 and ratio of p38 to pERK1/2, which was reversed by p38 inhibitor. Sosa and his colleagues [21] suggested a NR2F1-dependent dormancy via SOX-9/RARβ axis in

![Fig. 4 Effect of NR2F1 silencing on the dormancy, migration and invasion of SACC-83 and SACC-LM cells. (a) CCK8 assay was used to examine the cell growth rates in control, NR2F1 hi SACC cells, NR2F1 hi SACC cells + NR2F1 siRNA group of SACC-83 and SACC-LM, respectively. The data showed that NR2F1 siRNA can rescue the proliferation of cells. Error bars represent the mean ± SD of triplicate experiments. *p < 0.05. (b) Flow cytometry was used to examine the cell cycle in control, NR2F1 hi SACC cells, NR2F1 hi SACC cells + NR2F1 siRNA group of SACC-83 and SACC-LM, respectively. Representative figures of three independent experiments were shown. (c) Migration assay examined the cell migration ability in control, NR2F1 hi SACC cells, NR2F1 hi SACC cells + NR2F1 siRNA group of SACC-83 and SACC-LM, respectively. Representative figures of three independent experiments were shown. NR2F1 siRNA could reduce the migration ability of SACC cells, compared with NR2F1 hi SACC cells. The mean was derived from cell counts of 3 fields, and each experiment was repeated 3 times. Error bars represent the mean ± SD of triplicate experiments. *p < 0.05. (d) Invasion assay examined the cell invasion ability in control, NR2F1 hi SACC cells, NR2F1 hi SACC cells + NR2F1 siRNA group of SACC-83 and SACC-LM, respectively. Representative figures of three independent experiments were shown. NR2F1 siRNA could inhibit the invasion ability of SACC cells compared with NR2F1 hi SACC cells. The mean was derived from cell counts of 3 fields, and each experiment was repeated 3 times. Error bars represent the mean ± SD of triplicate experiments. *p < 0.05. (e) Flow cytometry showed cell apoptosis in control, NR2F1 hi SACC cells, NR2F1 hi SACC cells + NR2F1 siRNA group of SACC-83 and SACC-LM, respectively. Apoptotic analysis of SACC cells showed no difference in siNRF1 SACC cells and NR2F1 hi SACC cells. Representative figures of three independent experiments were shown]
HNSCC and breast cancer. Additionally, NR2F1 could induce global chromatin repression and act as a key gene which contributes to dormancy of DTCs in the bone marrow, while the effect of NR2F1 on growth arrest was reversed by siRNA or knockdown. The results further affirmed that NR2F1 was a critical node in dormancy induction.

The CXCL12/CXCR4 signaling is composed of the chemokine CXCL12 (also called SDF-1 for stromal cell-derived factor 1) and its receptors CXCR4 and CXCR7, playing pivotal roles in the cell migration, angiogenesis, proliferation, and survival of many cancer cells, including SACC [22, 23]. Here, we found that the high expression of NR2F1 promoted the expression of CXCL12 and CXCR4, and overexpression of CXCL12 rescued SACC cell behaviors inhibited by NR2F1 silencing. This is supported by the data of Boudot group, who detected that NR2F1 stimulated the metastatic cascade via CXCL12/CXCR4 pathway by activating epithelial growth factor (EGF) and EGF receptor in breast cancer [37]. This indicated that NR2F1 may contribute to cancer cell dormancy, invasion and metastasis of salivary adenoid cystic carcinoma by activating CXCL12/CXCR4 pathway.

Targeting the tumor dormancy is far from clinical application, but the NR2F1 regulation on tumor dormancy comprises several therapeutic insights both in clinical use and under clinical trials [36, 38]. William and his group have launched a clinical trial in combination treatment of 5-Aza and AtRA for patients with recurrent prostate cancer. 20 participants were randomly recruited and treated with reprogramming therapy, which utilizing a combination of 5-Aza and AtRA to elicit a NR2F1-regulatory cancer dormancy process. Although the results are waiting to be published, it is anticipated to decrease the rate of disease progression-free and to suffer a low percentage of adverse events.
Conclusions

Our data confirmed that NR2F1 could induce SACC cells into dormancy and high NR2F1 expression was strongly associated with increased lung metastatic potential. NR2F1 may serve as a valuable marker for cancer dormancy of SACC patients. Hence, we hypothesized that the permissive microenvironment of tumor growth in the lung may “wake up” these dormant tumor cells and suggested an underlying mechanism to explain high rate of lung metastasis formation in patients with SACC. These provided the promising advancements in our understanding of the SACC dormancy and genetically targeted therapies.
Abbreviations
ChIP: Chromatin immunoprecipitation; DIF staining: Double immunofluorescence staining; DTCs: Disseminated tumor cells; EGF: Epithelial growth factor; HE staining: Hematoxylin-eosin staining; HNSCC: Head and neck squamous cell carcinoma; NR2F1: Nuclear receptor subfamily 2 group F member 1; SACC: Salivary adenoid cystic carcinoma; SDF-1: Stromal cell-derived factor 1; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

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Authors’ contributions
XLG, XHL and YLT conceived and designed the project. LLD, XHY and XY participated in the collection of tissue samples. XLG, MZ1 and HFW performed the vivo and vitro assays. XP, LL and MZ2 analyzed the data. XLG and YLT were the major contributors in writing the manuscript. SSW, JBW and YJT revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
This is the case and the raw data can be requested from Dr. Tang Yaling and Miss Gao Xiaolei.

Ethics approval and consent to participate
The human specimens in this study were reviewed and approved by the Institutional Ethics Committee of the West China Medical Center, Sichuan University, No.14, Sec. 3, Renminnan Road, Chengdu 610041, Sichuan, China. Every patient signed separate informed consent forms for sampling and human data is in accordance with the principles of Declaration of Helsinki. University, China (No.WCHSIRB-D-2017-144). And the research involving Institution Ethics Committee of the West China Medical Center, Sichuan University, No.739, Dingshen Road, Lincheng Street, Zhoushan, Zhejiang Province, China grants (grant nos. 81772881, 81672672, 81572650, and 81502357), Natural Science Foundation of Zhejiang Province (grant no. Q14114001), Zhoushan Science and Technology Bureau Project (grant no. 2014C3106) and by National Program on Key Research Project of China (grant no. 2016YFC0902700). All the funds in this article were used for the design of the study and for the collection, analysis and interpretation of the data and writing of the manuscript. No special funding.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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