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Original Article

Protein inhibitor of activated signal transducer and activator of transcription 2 is an oncoprotein in oral squamous cell carcinoma and related to cigarette smoking - An in vitro study

Szu-Yu Chen [a], Chi-Fu Chiang [a], Yu-Fu Su [b], Che-Yi Lin [c*], Yi-Jen Hung [d**], Tsai-Wang Huang [e***], Yi-Shing Shieh [f,g,h****]

[a] *Graduate Institute of Medical Sciences, National Defense Medical Center, Taipei, Taiwan*
[b] *Department of Radiation Oncology, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan*
[c] *Department of Oral and Maxillofacial Surgery, Chi Mei Medical Center, Tainan, Taiwan*
[d] *Division of Endocrinology and Metabolism, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan*
[e] *Division of Thoracic Surgery, Department of Surgery, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan*
[f] *Department and Graduate Institute of Biochemistry, National Defense Medical Center, Taipei, Taiwan*
[g] *School of Dentistry, College of Dental Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan*
[h] *Department of Dentistry, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan*

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* Corresponding author. Department of Oral and Maxillofacial Surgery, Chi Mei Medical Center, No.901, Zhonghua Rd, Yongkang District, Tainan, 710, Taiwan.
** Corresponding author. Division of Endocrinology and Metabolism, Tri-Service General Hospital, National Defense Medical Center, No.161, Sec. 6, Min-Chuan East Rd., Nei-Hu, Taipei, 114, Taiwan.
*** Corresponding author. Division of Thoracic Surgery, Department of Surgery, Tri-Service General Hospital, National Defense Medical Center, No.161, Sec. 6, Min-Chuan East Rd., Nei-Hu, Taipei, 114, Taiwan.
**** Corresponding author. School of Dentistry, Tri-Service General Hospital, National Defense Medical Center, No.161, Sec. 6, Min-Chuan East Rd., Nei-Hu, Taipei, 114, Taiwan.
E-mail addresses: d920706@mail.chimei.org.tw (C.-Y. Lin), metahung@yahoo.com (Y.-J. Hung), chi-wang@mail.ndmctsgh.edu.tw (T.-W. Huang), ndmcyss@ndmctsgh.edu.tw (Y.-S. Shieh).

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KEYWORDS
Cigarette smoke condensate;
Epithelial-mesenchymal transition;
Oncoprotein;
Oral squamous cell carcinoma;
Protein inhibitor of activated STAT 2

Abstract *Background/purpose:* Oral cancer is one of the most prevalent malignant tumors in Taiwan. Due to the heterogeneity of oral cancer cells, the five-year survival rate of patients is only 50%. Post-translational modifications contribute to protein diversity and directly influence cell functions. The protein inhibitor of activated signal transducer and activator of transcription 2 (PIAS2) is known to undergo post-translational modifications, yet its impact on oral cancer remains unclear.
Materials and methods: PIAS2 expression was modulated by transfecting cells with a PIAS2 expression vector or by knocking down PIAS2 using siRNA with low and high PIAS2 expression, respectively. These cells were subjected to invasion, migration, and proliferation assays to evaluate the effects of PIAS2. Changes in genotype, such as epithelial-mesenchymal transition (EMT) markers, were also examined. Additionally, the effect of cigarette smoke condensate (CSC) on PIAS2 expression in oral squamous cell carcinoma (OSCC) cells was investigated.
Results: Overexpression of PIAS2 significantly increased the malignant behaviors of oral cancer cells. In YD38 and SAS cells with low PIAS2 expression, overexpression of PIAS2 enhanced proliferation, invasion, and migration. PIAS2 overexpression also affected EMT gene expression, suppressing E-cadherin and increasing fibronectin expression. Conversely, PIAS2 knockdown in OECM1 and SCC25 cells suppressed malignant behaviors and reversed EMT markers, increasing E-cadherin and decreasing fibronectin expression. Furthermore, a dose-dependent increase in PIAS2 expression was observed when OSCC cells were treated with CSC.
Conclusion: PIAS2 functions as an oncogene in oral cancer, and cigarette smoking induces PIAS2 expression. Increased PIAS2 levels lead to enhanced malignancy in oral cancer.

Introduction

Oral cancer is the sixth most common type of cancer worldwide, and the most common cancer of head and neck tumors.[1] Above all the oral cancers, oral squamous cell carcinoma (OSCC) is the most common one, accounting for 90% of cases.[2] The standard treatment for oral cancer includes surgery, chemotherapy, and radiotherapy.[3] However, the therapeutic outcomes remain unsatisfactory, with only half of the patients surviving beyond five years.[4] This poor prognosis is attributed to the heterogeneity of cancer cells, rapid disease progression, and a high propensity for metastasis.[4] Therefore, identifying therapeutic targets and prognostic indicators has become a critical area of research.

The protein inhibitor of activated signal transducer and activator of transcription 2 (PIAS2) is a member of the protein inhibitor of activated STAT family, which can inhibit the JAK-STAT signaling pathway.[5] PIAS2, for instance, has been shown to suppress STAT4-dependent gene induction associated with IL-12.[6] Additionally, PIAS proteins can undergo post-translational modifications as specific E3 SUMO ligases.[7] PIAS2 is known to SUMOylate the p53 tumor suppressor and c-Jun, thereby repressing p53 activity and regulating other transcription factors.[8] PIAS2 also SUMOylates the androgen receptor (AR), a member of the steroid receptor superfamily.[9] Furthermore, PIAS2 can influence cellular capabilities by altering cell mobility and morphology.[10] PIAS2 SUMOylates actin, tubulin, and myosin, affecting cytoskeleton organization and cell motility.[10] PIAS2 knockout cells lose their ability to form pseudopodia, resulting in a more rounded shape and altered cell morphology.[11] These findings demonstrate that PIAS2 exerts its effects at multiple cellular levels.

PIAS2 also plays a significant role in cancer. As a tumor suppressor, PIAS2 can inhibit the ubiquitination of phosphatase and tensin homolog (PTEN), suppressing the proliferation of sarcoma and cancer cell lines.[7] In osteosarcoma, increased levels of PIASxα were associated with reduced expression of cyclin D1 and cyclin D3, indicating PIASxα's repressive effects on the cell cycle.[12] Additionally, PIAS2 enhances the transcriptional activity of peroxisome proliferator-activated receptor-gamma (PPAR-γ), inducing apoptosis in HepG2 hepatoma cells.[13] Conversely, PIAS2 can function as an oncoprotein that promotes cancer progression. PIAS2 enhances the SUMOylation of smad nuclear interacting protein 1 (SNIP), thereby reducing the repression of TGF-β and NF-κB signaling, which induces the migration and invasion of lung cancer epithelial cells (A549) and keratinocyte cells (HaCaT).[14] In acute promyelocytic leukemia, PIAS2α mediates promyelocytic leukemia protein (PML) SUMOylation and promotes replicative senescence.[15] Furthermore, PIAS2 SUMOylates the transcription factor Zinc finger homeobox 3 (ZFHX3), increasing the proliferation and stability of breast cancer cells.[16] These studies suggest that PIAS2's functions may vary depending on the cellular and tissue context. However, the role of PIAS2 in oral squamous cell carcinoma remains unknown, necessitating further research.

In this article, we demonstrated that PIAS2 exacerbated the malignancy of oral squamous cell carcinoma by increasing the proliferation, invasion, and migration

abilities of oral cancer cells. Additionally, PIAS2 influenced epithelial-mesenchymal transition (EMT) markers, making cancer cells more prone to carry out metastasis.

Materials and methods

Cell line and cell culture

The human OSCC cell lines, OECM1, YD38 (gingiva carcinoma), and SAS, SCC25 (tongue carcinoma), were grown in Roswell Park Memorial Institute (RPMI) medium (GIBCO, Eggenstein, Germany) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Eggenstein, Germany) and 1% Penicillin-Streptomycin-Amphotericin B (PSA) (Biological Industries, Cromwell, CT, USA). The OECM1 cells were obtained from Merck Millipore, Darmstadt, Germany. The SAS cells were kindly provided by Dr. Michael Hsiao at Genomics Research Center, Academia Sinica, Taipei, Taiwan. The SCC25 cells were kindly provided by Dr. Shine-Gwo Shiah (National Institute of Cancer Research, National Health Research Institutes, Miaoli, Taiwan). The YD38 cells were obtained from Dr. Yook (Namseoul University, Seoul, Korea).

Drugs and antibodies

Cigarette smoke condensate (CSC) (NC1560725, Murty Pharmaceuticals, Lexington, KY, USA) was prepared by the standard of 3R4F cigarettes, a composition from University of Kentucky's. The primary antibodies used were as follows: PIAS2 (Santa Cruz, sc-166494, Dallas, TX, USA), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell signaling, 5174, Danvers, MA, USA), E-cadherin (Cell Signaling, 14472), Fibronectin (Abcam, ab32419, Cambridge, MA, USA), Anti-rabbit or anti-mouse secondary antibodies were purchased from Jackson Immuno Research Laboratories (West Grove, PA, USA). α-Tubulin and Lamin B1 antibodies were obtained from Proteintech (Wuhan, China).

Plasmid transfection and rna interference

In this study, the PolyJetTM in Vitro DNA Transfection Reagent (Cat. #SL100688) from SignaGen Laboratories was used for transfection experiments. SiRNAs were synthesized by Dharmacon (Lafayette, CO, USA). We add 3ul PolyJetTM Reagent and 1ug plasmid DNA (pCMV-Tag2A and pCMV-Tag2A-PIAS2) are respectively diluted with 50ul serum-free culture medium, then add the diluted PolyJetTM Reagent to the diluted plasmid DNA, mix well, and stand still 15 min to form a PolyJetTM/DNA complex, and finally add the complex to the culture dish and incubate for 16 h. Transfection of siRNA (10 nM) into cells was performed using GenMute siRNA transfection reagent (SignaGen Laboratories, Ijamsville, MD, USA) following the manufacturer's instructions.

RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was generated by reverse transcription of total RNA using a High-Capacity cDNA Synthesis kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was quantified using a NanoDrop Spectrophotometer (NanoDrop, Wilmington, DE, USA). The expressions of related genes in our study were analyzed using quantitative real-time polymerase chain reaction (RT-PCR). Quantitative RT-PCR was performed on a Roche LightCycler 480 system with SYBR Green I Master mix (Roche, Indianapolis, IN, USA). The conditions were as follows: an initial heat denaturation at 95 °C for 2 min, followed by 40 cycles at 95 °C for 5 s and 60 °C for 20 s. GAPDH was used as an internal control. The primer sequences are listed.

PIAS2 forward: CCGGAATTCAATGGCGGATTTCGAAGGAGTTG, PIAS2 reverse: CCCAAGCTTTTAGTCCAATGAGATGATGTCAG, GAPDH forward: CCACATCGCTCAGACACCAT, GAPDH reverse: TGACCAGGCGCCCAATA.

Western blot analysis

Protein lysates were extracted by RIPA buffer containing protease (Biological industries, Cromwell, CT, USA) and phosphatase (Biological industries, Cromwell, CT, USA) inhibitors. Nuclear and cytoplasmic fractions were extracted by NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher, Pittsburgh, PA, USA) according to the manufacturer's instructions. Protein concentration was determined using a Pierce BCA protein assay kit (Thermo Fisher, Pittsburgh, PA, USA). Equal amounts of protein samples were electrophoretically separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Darmstadt, Germany). The membranes were blocked with 5% non-fat milk for 1 h at room temperature and then incubated with primary antibodies diluted with blocking buffer overnight at 4 °C. The next day, the membranes were washed with 0.1% TBST and subsequently incubated with HRP—conjugated secondary antibodies for an additional 1 h at room temperature. Protein bands were visualized using ECL reagents (Merck Millipore, Darmstadt, Germany) and captured by a chemiluminescence image system (UVP Inc., San Gabriel, CA, USA).

Wound healing assay

Cell migration ability was determined using a wound-healing assay. Cells were plated and grown to confluence in six-well plates. The confluent cell monolayer of each well was scratched using a sterile micropipette tip to create a wound. After washing with Phosphate buffered saline (PBS) to remove floating debris, the cells were

cultured for an additional 18 h. Closure of the wounded areas was observed using an inverted microscope and photographed at 0 and 12 h. ImageJ software was used to quantify the wound area. The migratory ability was calculated by the area reduction at 12 h compared to the wound area at 0 h.

Transwell invasion assay

Cell invasion ability was analyzed using a Transwell assay, which was carried out in 24-well plates using Transwell chambers with an 8-μm pore size (Millipore, Billerica, MA, USA) pre-coated with Matrigel (Corning Inc., Corning, NY, USA). Cells were seeded at 2.5×10^4 in the upper chamber of the insert. After 24 h incubation, the invaded cells on the bottom side of the membrane were fixed with 100% methanol, stained with propidium iodide (PI), and photographed. The average number of invaded cells were counted from five microscopic fields per chamber of three independent experiments using ImageJ software.

Colony formation assay

Cell proliferation was assessed using colony formation assay. Oral cancer cells were seeded at a density of 1000 cells per well in 6-well plates and incubated in a cell culture incubator for 7 days. After incubation, the cells were stained with a 1% crystal violet solution for 30 min on a shaker, followed by washing off the excess dye with water. The number of colonies was then quantified using ImageJ software.

Statistical analysis

The Student's t-test was used to determine statistically significant differences between the two groups. All statistical analyses were performed using GraphPad Prism 8.0 programs (GraphPad Software, San Diego, CA, USA). Data were represented as the means ± SEM. The *P* value less than 0.05 was regarded as being statistically significant.

Results

Overexpression of PIAS2 increases malignancy in oral cancer cells

To investigate the impact of PIAS2 in oral cancer, we examined a panel of oral cancer cell lines with varying levels of PIAS2 expression. The selected cell lines belong to oral squamous cell carcinoma, specifically gingival (SCC25 and YD38) and tongue (OECM1 and SAS) carcinomas. Western blot analysis was used to detect the protein expression of PIAS2. As shown in Fig. 1, PIAS2 levels were higher in SCC25 compared to YD38, and similarly higher in OECM1 compared to SAS (Fig. 1A and B). We then overexpressed PIAS2 in SAS and YD38 cell lines and knocked down PIAS2 expression in SCC25 and OECM1 cell lines to further evaluate the effect of PIAS2 regulation on the genotype and malignancy of oral cancer.

Compared to the vector group, the mRNA expression of PIAS2 increased in PIAS2-transfected cells (Fig. 1C). Protein levels of PIAS2 also increased after transfection in YD38 and SAS cells (Fig. 1D). First, we examined the impact of PIAS2 on EMT. We used E-cadherin as an epithelial marker and fibronectin as a mesenchymal marker. When PIAS2 expression increased, E-cadherin expression decreased while fibronectin expression increased (Fig. 1E). This indicates that PIAS2 promotes the transition of oral cancer cells from an epithelial to a mesenchymal state, potentially increasing their malignancy.

Next, we conducted functional assays to assess the effect of PIAS2 on oral cancer cell lines. A wound healing assay demonstrated that increased PIAS2 expression enhanced the migration ability of SAS and YD38 cells (Fig. 2A and B). Overexpression of PIAS2 also increased invasion ability, as shown by the transwell invasion assay (Fig. 2C and D). Additionally, proliferation ability and colony numbers increased with PIAS2 overexpression (Fig. 2E and F).

Knockdown of PIAS2 expression in oral cancer cell lines

We used siRNA to knock down PIAS2 expression in OECM1 and SCC25 cell lines. Compared to the scramble control, PIAS2 mRNA expression decreased in the siRNA-PIAS2 group (siPIAS2) (Fig. 3A), and PIAS2 protein expression also decreased following siPIAS2 transfection (Fig. 3B). We verified the effects on EMT in knockdown cells. As PIAS2 expression decreased, E-cadherin expression increased and fibronectin expression decreased (Fig. 3C).

Functional assays further demonstrated that PIAS2 knockdown reduced the migratory ability of OECM1 and SCC25 cells (Fig. 4A and B). PIAS2 knockdown also decreased invasion ability, as shown by the transwell invasion assay (Fig. 4C and D). Additionally, proliferation ability decreased in the colony formation assay (Fig. 4E and F).

Cigarette smoke condensate increases PIAS2 expression

After confirming the malignant effects of PIAS2, we investigated factors influencing PIAS2 expression. We found that CSC increases PIAS2 expression in a dose-dependent manner (Fig. 5A and B). This phenomenon was observed in both YD38 and SAS cell lines, suggesting a direct relationship between PIAS2 expression and smoking.

Discussion

In this article, we demonstrated that PIAS2 is an oncoprotein in OSCC. Through overexpression and knockdown experiments, we revealed that PIAS2 induces EMT changes and enhances malignancy in oral cancer cells. PIAS2 leads to an overall increase in cell invasion, migration, and proliferation. This is the first report highlighting PIAS2 in oral cancer, and further investigation to validate its clinical relevance is necessary.

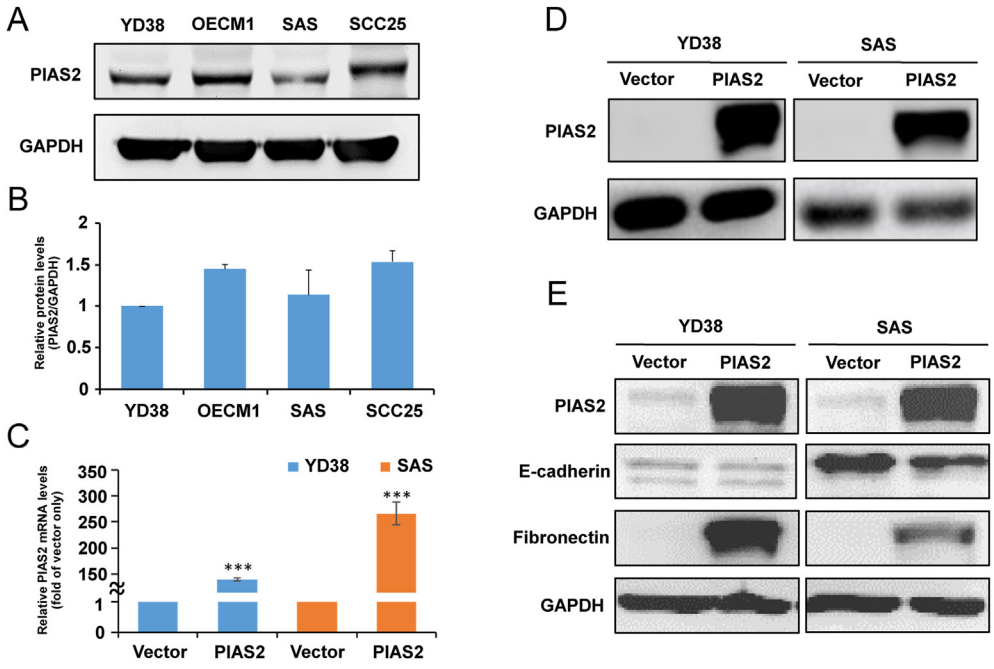

Figure 1 Overexpression of PIAS2 in oral cancer increases epithelial-mesenchymal transition. (A) Endogenous protein levels in oral cancer cell lines (YD38, OECM1, SAS, and SCC25). The expression of PIAS2 protein in YD38 and OECM1 (gingiva squamous cell carcinoma), and SAS and SCC25 (tongue oral squamous carcinoma cell lines) was examined using the Western blot method. (B) Quantitative results of the PIAS2 Western blot analysis. (C) PIAS2 expression in YD38 and SAS cell lines was increased by transfecting with 1 μg of pCMV Tag2A-PIAS2 in a six-well plate. After 48 h of incubation, mRNA and protein were extracted for analysis. Changes in PIAS2 mRNA levels were analyzed using qRT-PCR. (D) Changes in PIAS2 protein levels were assessed by Western blot. (E) PIAS2 expression affects EMT protein levels in oral cancer cell lines. In a six-well plate, 1 μg of PIAS2 plasmid was used to overexpress PIAS2 in YD38 and SAS cell lines. After 48 h of incubation, protein was extracted. Changes in mesenchymal marker proteins (Fibronectin) and epithelial marker proteins (E-cadherin) were assessed by Western blot. Data are shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. PIAS2, Protein inhibitor of activated signal transducer and activator of transcription 2; EMT, epithelial-mesenchymal transition; SEM, standard error of the mean.

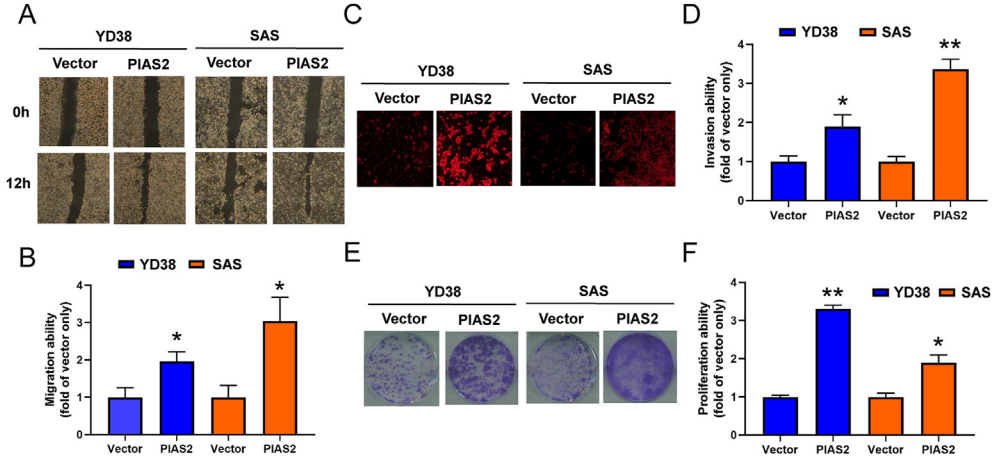

Figure 2 Overexpression of PIAS2 increases migration, invasion, and proliferation of human oral cancer cells. (A) Migratory ability was evaluated using a wound-healing assay. Representative images were taken at 0 and 12 h after wound scratching. Wound closure was quantified using ImageJ software. (B) Quantitative results of the wound-healing assay. (C) Invasion ability was evaluated using a transwell invasion assay. OSCC cells were treated as indicated and allowed to invade through matrigel-coated inserts for 24 h. PI-stained cells were quantified using ImageJ software. (D) Quantitative results of the invasion assay. (E) Proliferation ability was evaluated using a colony formation assay. 1000 cells were plated in six-well plates and incubated for 7 days. Crystal violet-stained colonies were quantified using ImageJ software. (F) Quantitative results of the colony formation assay. Data are shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. PIAS2, Protein inhibitor of activated signal transducer and activator of transcription 2; OSCC, Oral squamous cell carcinoma; SEM, standard error of the mean.

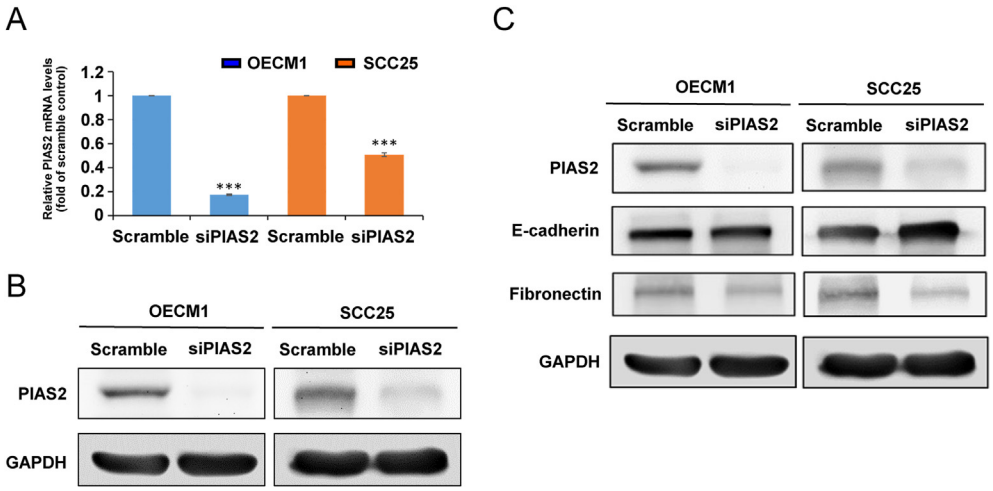

Figure 3 Knockdown of PIAS2 in oral cancer reverses epithelial-mesenchymal transition. (A) PIAS2 expression in OECM1 and SCC25 cell lines was decreased by transfecting with 10 nM siPIAS2 in a six-well plate. After 48 h of incubation, mRNA and protein were extracted for analysis. Changes in PIAS2 mRNA levels were analyzed using qRT-PCR. (B) Changes in PIAS2 protein levels were assessed by Western blot. (C) PIAS2 expression affects EMT protein levels in oral cancer cell lines. In a six-well plate, 10 nM siPIAS2 was used to reduce PIAS2 expression in OECM1 and SCC25 cell lines. After 48 h of incubation, protein was extracted. Changes in mesenchymal marker proteins (Fibronectin) and epithelial marker proteins (E-cadherin) were assessed by Western blot. Data are shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. PIAS2, Protein inhibitor of activated signal transducer and activator of transcription 2; EMT, epithelial-mesenchymal transition; SEM, standard error of the mean.

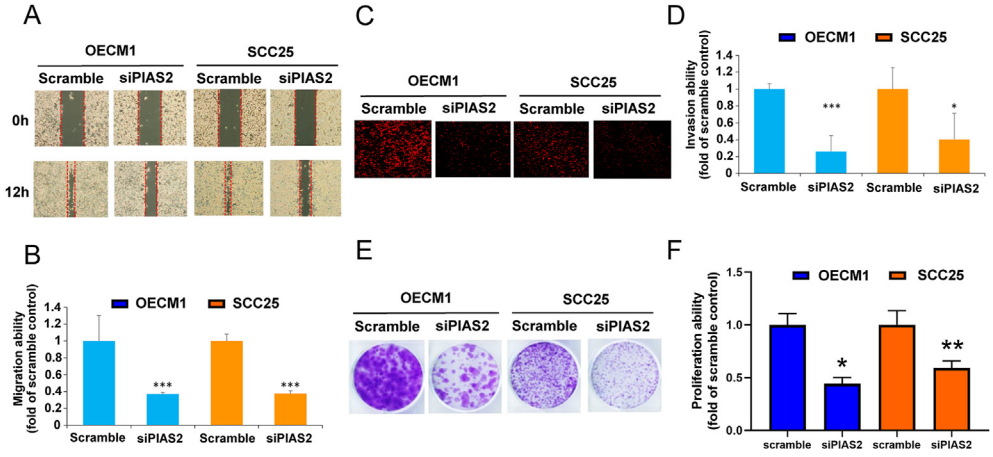

Figure 4 Knockdown of PIAS2 decreases malignant behaviors of human oral cancer cells. (A) Migratory ability was evaluated using a wound-healing assay. Representative images were taken at 0 and 12 h after wound scratching. Wound closure was quantified using ImageJ software. (B) Quantitative results of the wound-healing assay. (C) Invasion ability was evaluated using a transwell invasion assay. OSCC cells were treated as indicated and allowed to invade through matrigel-coated inserts for 24 h. PI-stained cells were quantified using ImageJ software. (D) Quantitative results of the invasion assay. (E) Proliferation ability was evaluated using a colony formation assay. 1000 cells were plated in six-well plates and incubated for 7 days. Crystal violet-stained colonies were quantified using ImageJ software. (F) Quantitative results of the colony formation assay. Data are shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. PIAS2, Protein inhibitor of activated signal transducer and activator of transcription 2; OSCC, Oral squamous cell carcinoma; SEM, standard error of the mean.

Additionally, our results align with previous reports indicating that PIAS2 contributes to malignancy in other cancers.[14–20] For example, PIAS2 promotes tumorigenesis in acute promyelocytic leukemia by degrading PML,[15] a factor also associated with oral stratified epithelium and its carcinogenesis.[17] In breast cancer, PIAS2 increases proliferation and protein stability by SUMOylating ZFHX3,[16] which is frequently upregulated in OSCC.[18] Moreover, PIAS2 enhances invasion and migration in lung cancer by increasing the SUMOylation of SNIP.[14] Smad expression, the primary regulated protein of SNIP, significantly increases from oral epithelial dysplasia to OSCC,[19] and is elevated in various types of odontogenic tumors.[20] The findings from these studies on PIAS2 will provide valuable references for further research into the mechanisms by which PIAS2 acts as an oncogene in oral cancer.

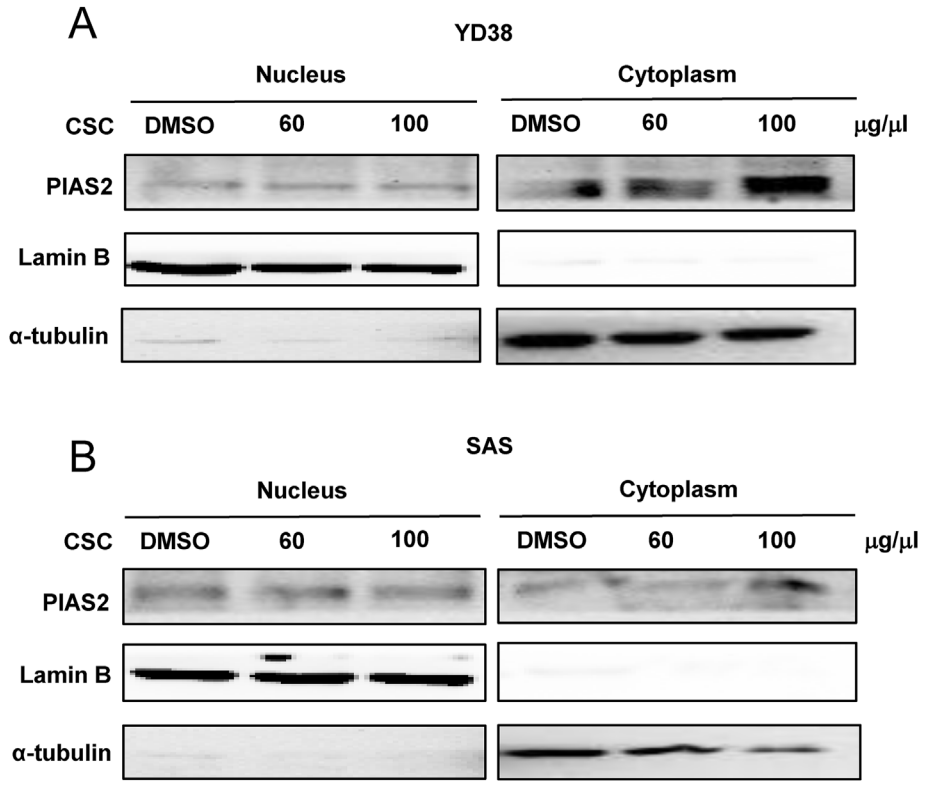

Figure 5 CSC treatment increases PIAS2 expression in oral cancer cells.
Dose-dependent changes in PIAS2 expression were observed after treatment with CSC in (A) YD38 and (B) SAS cells. CSC, cigarette smoke condensate; PIAS2, Protein inhibitor of activated signal transducer and activator of transcription 2.

Our data suggest a dose-dependent relationship between CSC and PIAS2. Previous studies have shown that CSC directly leads to the occurrence of epithelial cancers, such as oral and lung cancer.[21,22] CSC contains numerous toxic and reactive compounds that can directly or indirectly influence the activity of transcription factors. For example, activation of NF-κB, AP-1, or other transcription factors by CSC components could lead to increased PIAS2 expression.[23] Chronic exposure to cigarette smoke elicits a persistent inflammatory response in the respiratory system. Besides affecting the epithelium, CSC also impacts smooth muscle cells and induces the production of inflammatory cytokines.[24] Additionally, cytokines such as TNF or interleukin, which are upregulated in response to cigarette smoke, could promote PIAS2 expression through signaling pathways like JAK/STAT.[25] CSC can influence epithelial cell behavior through miRNA,[26] and also stimulates SUMOylated substrates and SUMOylation levels in chronic obstructive pulmonary disease.[27] The mechanism behind the increase in PIAS2 along with CSC exposure remains to be identified, but it may be a contributing factor to cancer development and progression.

However, our current research is focused on in vitro studies. To further validate PIAS2 in oral cancer progression, animal and human specimen experiments are necessary. For example, subcutaneously injecting oral cancer cells into mice to further analyze tumor size, weight, and changes in PIAS2 expression influenced by CSC. Additionally, analyzing the PIAS2 and its association with clinicopathological features in oral cancer patients with or without smoking history could help us to clarify its role in oral cancer.

In conclusion, we found that PIAS2 is an oncoprotein in OSCC. PIAS2 increases the migration, invasion, and proliferation capabilities of oral cancer cell lines. It also promotes EMT and accelerates cancer progression. Although the precise mechanisms are still under investigation, PIAS2 remains a potential biomarker and therapeutic target.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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