p12$^{CDK2-AP1}$ interacts with CD82 to regulate the proliferation and survival of human oral squamous cell carcinoma cells

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Abstract. p12 cyclin-dependent kinase 2 (CDK2)-associating protein 1 (p12$^{CDK2-AP1}$) has been demonstrated to negatively regulate the activity of CDK2. However, the underlying molecular mechanism remains largely unknown. We aimed to determine the potential binding proteins of p12$^{CDK2-AP1}$ and to elucidate the role of p12$^{CDK2-AP1}$ in the regulation of the proliferation, invasion, apoptosis, and *in vivo* growth of human oral squamous cell carcinoma cells. The protein-protein interaction was predicted using computational decision templates. The predicted p12$^{CDK2-AP1}$ interacting proteins were overexpressed in human oral squamous cell carcinoma OSCC-15 cells, and the protein binding was examined using co-precipitation (Co-IP). Cell proliferation and invasion were determined via MTT assay and Transwell system, respectively. Cell apoptosis was evaluated using Annexin V-FITC/PI double staining followed by flow cytometric analysis. The *in vivo* growth of OSCC-15 cells was examined in nude mouse tumor xenografts. We found that overexpression of either p12$^{CDK2-AP1}$ or CD82 significantly suppressed the proliferation and invasion but promoted the apoptosis of OSCC-15 cells ($P<0.05$). Importantly, combined overexpression of p12$^{CDK2-AP1}$ and CD82 showed synergistic antitumor activity compared with the overexpression of a single protein alone ($P<0.05$). Additionally, the simultaneous overexpression of p12$^{CDK2-AP1}$ and CD82 significantly suppressed the *in vivo* tumor growth of OSCC-15 cells in nude mice compared with the negative control ($P<0.05$). Our findings indicate that p12$^{CDK2-AP1}$ interacts with CD82 to play a functional role in suppressing the *in vitro* and *in vivo* growth of OSCC-15 cells.

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Introduction

Head and neck cancer represents the sixth most common human cancer and includes cancers of the lip, oral cavity (mouth), nasal cavity (inside the nose), paranasal sinuses, pharynx, and larynx (1). Approximately 90% of head and neck cancers are squamous cell carcinoma (HNSCC), which is the leading malignancy in humans (2). An estimated 300,400 new cases and 145,400 deaths from oral cavity cancers occurred in 2012 worldwide (3). The development of HNSCC is a result of multiple genetic and epigenetic alterations, including the activation of oncogenes and the loss of function of tumor-suppressor genes (4). Nevertheless, the precise mechanism of the tumorigenesis in HNSCC remains largely unknown.

p12, a cyclin-dependent kinase 2 (CDK2)-associating protein 1 (p12$^{CDK2-AP1}$), was initially identified as a cancer-related gene in a hamster oral cancer model (5). The human p12$^{CDK2-AP1}$ gene locates on chromosome 12q24 and encodes a 115-amino acid protein (6). In human oral squamous cell carcinoma (OSCC), p12$^{CDK2-AP1}$ functions as a tumor suppressor by negatively regulating the activity of CDK2, which plays a crucial role in mediating cell cycle progression (7,8). In addition to CDK2, p12$^{CDK2-AP1}$ has been found to interact with other proteins including DNA polymerase α/primase (9) and its homologous protein p14 (10). Protein interaction plays a pivotal role in the control of various essential biological processes; thus, it is important to identify and validate other p12$^{CDK2-AP1}$ interacting proteins to understand its role in cell cycle regulation and tumorigenesis.

Computational approaches have been developed for the large-scale prediction of protein-protein interaction based on protein sequence, structure and evolutionary relationships in complete genomes in attempts to unravel the global protein interactome (11-13). In the present study, a multiple and pairwise kernel support vector machine was employed to predict the interactive proteins of p12$^{CDK2-AP1}$. CD82 was identified to be a protein with high binding affinity to p12$^{CDK2-AP1}$. We further found that the interaction between p12$^{CDK2-AP1}$ and

CD82 played a synergistic role in suppressing the tumorigenesis and development of OSCC, suggesting that the interference of p12$^{CDK2-AP1}$/CD82 interaction may provide a valuable strategy for managing OSCC.

Materials and methods

Prediction of protein-protein interactions (PPIs). To predict the interacting proteins of p12$^{CDK2-AP1}$, we employed a multiple and pairwise kernel support vector machine as previously described (11,14). With these approaches, the one- and two-dimensional structures as well as the eigenvector of p12$^{CDK2-AP1}$ were simulated. A total of 10 proteins from the Swiss-Port database and the potential PPIs were predicted based on the decision template.

Reagents. PCR primers were synthesized by Sangon Biotech (Shanghai, China). The pIRES2-EGFP vector was purchased from YouBio (Changsha, China). Dulbecco's modified Eagle's medium (DMEM) and Opti-MEM® medium (low serum culture medium) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Invitrogen (Carlsbad, CA, USA), respectively. Fetal bovine serum (FBS) was purchased from Gibco (New York, NY, USA). Six-well Transwell polycarbonate filters (8-μm pore size) were purchased from Millipore (Boston, MA, USA). Matrigel was obtained from BD Biosciences (New York, NY, USA). Annexin V-FITC was purchased from BLKW Biotechnology (Beijing, China).

Construction of the recombinant plasmids. The full-length coding sequences of p12$^{CDK2-AP1}$ and CD82 were amplified from the human brain library using PCR. The sequences of the PCR primers were as follows: p12$^{CDK2-AP1}$ forward, 5'-ATGTCT TACAAACCGAACTTGG-3' and reverse, 3'-GGATCTGGCA TTCCGTTCCG-5'; and CD82 forward, 5'-ATGGGCTCAGCC TGTATCAAAG-3' and reverse, 3'-GTACTTGGGGACCTTG CTGTA-5'. The amplified products were inserted into the pIRES2-EGFP vector to obtain pIRES2-EGFP-p12$^{CDK2-AP1}$ and pIRES2-EGFP-CD82 plasmids. The sequences of the plasmids were confirmed via direct DNA sequencing.

Cell culture and transfection. Both human embryonic kidney 293T cells and human oral squamous cell carcinoma OSCC-15 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in DMEM supplemented with 10% FBS at 37˚C in a humidified atmosphere with 5% CO_2. The cultured cells were passaged every two days with fresh culture medium. 293T and OSCC-15 cells were transfected with the pIRES2-EGFP-p12$^{CDK2-AP1}$, pIRES2-EGFP-CD82, or pIRES2-EGFP negative control (NC) plasmids using Lipofectamine™ 2000 transfection reagent in Opti-MEM® medium according to the manufacturer's recommendations (Invitrogen). The protein levels of p12$^{CDK2-AP1}$ and CD82 were examined using western blot analysis at 0, 24, 48, and 72 h after transfection. Fluorescence was examined under a fluorescence microscope (BX51-32FB3F01; Olympus, Japan).

Western blot analysis. Total proteins were extracted from cultured cells using RIPA lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS in 1X phosphate buffer solution) containing protease inhibitor (2 μg/ml aprotinin, 2 μg/ml leupeptin and 1 M PMSF) for 30 min on ice. After centrifuging, the supernatant was resuspended in buffer containing 1% SDS and 1% dithiothreitol and heated at 100˚C for 5 min. Equal amounts of proteins were electrophoresed using 10% SDS-PAGE and then transferred onto nitrocellulose membranes (#88018; Pierce, USA). After being blocked with 5% non-fat dry milk in Tris-buffered saline and Tween-20 (10 mM Tris-HCl, pH 8.0, 100 mM NaCl and 0.05% Tween, TBS-T), the membrane was incubated at 4˚C overnight with the mouse polyclonal anti-CDKA1 (1:200, ab167256), mouse monoclonal anti-CD82 (1:200, ab140238) (both from Abcam, Cambridge, UK) or mouse polyclonal anti-GAPDH (1:800; Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies. After incubation, the membrane was washed twice with TBS-T for 15 min and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Abcam). After TBS-T washing, the immunoreactivities were visualized using an enhanced chemiluminescence (ECL) kit according to the manufacturer's instructions (Abcam). The relative optical density of the bands of interest was analyzed using Image-Pro Plus 6.0 software.

Co-immunoprecipitation (Co-IP). OSCC-15 cells were lysed in IP cell lysis buffer (0.6 ml 1 M Tris-HCl pH 6.8, 5 ml 50% glycerinum, 2 ml 10% SDS, 0.5 ml 2-mercaptoethanol, 1 ml 1% bromophenol blue, 0.9 ml distilled water) (P0013; Beyotime, Shanghai, China) containing protease inhibitors (Roche). The whole cell lysates were incubated with an antibody together with 20 μl Protein A Plus agarose beads (#20333; Pierce) overnight at 4˚C. The immunoprecipitates were washed three times with IP cell lysis buffer, resuspended in 20 μl of 2X SDS loading buffer, heated at 100˚C for 10 min and then loaded for SDS-PAGE electrophoresis and western blotting using a goat anti-mouse IgG/HRP.

MTT assay. To determine the proliferation capability of the cells, an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay was performed. Non-transfected 293T cells (OSCC-15) and cells transfected with NC plasmid (OSCC-15+NC), p12$^{CDK2-AP1}$ (OSCC-15+p12$^{CDK2-AP1}$), CD82 (OSCC-15+CD82) or p12$^{CDK2-AP1}$ plus CD82 (OSCC-15+p12$^{CDK2-AP1}$+CD82) were used for the MTT assay. The cells were seeded in 96-well plates at a density of 1x10^4 cells/well and maintained at 37˚C in a humidified atmosphere with 5% CO_2 overnight. After incubation, 20 μl of MTT reagent (5 mg/ml) was added to each well followed by another 4 h of incubation. The MTT solution was removed, and dimethyl sulfoxide (DMSO) was added to each well to dissolve the metabolic product. The absorbance at 570 nm was recorded. The relative proliferation rate of the cells was calculated according to the following equation: Relative proliferation rate of the cells = (Absorbance value of the sample/Absorbance value of the control) x 100%. Data were calculated from three independent experiments.

Determination of the cell invasive capability. Cell invasive ability was determined using a 6-well Transwell system. In brief, 50 mg/l of Matrigel was diluted in serum-free medium

to a final concentration of 3.9 μg/µl. After that, 60-80 μl of Matrigel was added onto the polycarbonate membrane and air-dried for 24 h. The transfected OSCC-15 cells were trypsinized, washed in PBS and suspended in 0.1% serum-containing medium. Approximately $5x10^4$ cells were loaded into the upper chamber wells and incubated at 37˚C in 5% CO_2 for 18 h. After incubation, the cells that had traversed the filter were fixed with methanol, washed in PBS, stained with hexamethyl pararosaniline and counted. The average number of invaded cells was calculated from 3 independent experiments.

Evaluation of cell apoptosis. OSCC-15 cells were collected at 72 h after transfection. Non-transfected cells were used as a control. The cells were washed twice with cold PBS, and approximately $1x10^5$-$1x10^6$ cells were re-suspended in 100 μl solution containing 5% Annexin V-FITC and incubated at 37˚C for 15 min in the dark. After PI labeling, the cells were analyzed using flow cytometry (BD Biosciences). The experiments were performed in triplicate. The early apoptosis rate (%) and the overall cell apoptosis rate (%) (early plus late) were calculated.

Tumor formation in nude mice. To understand the influences of $p12^{CDK2\text{-}AP1}$ and CD82 on *in vivo* tumor formation, non-transfected cells (OSCC-15), cells transfected with the NC plasmid (OSCC-15+NC), or the $p12^{CDK2\text{-}AP1}$ plus CD82 (OSCC-15+$p12^{CDK2\text{-}AP1}$+CD82) plasmids were subcutaneously inoculated into nude mice. Sixty-three 5-week-old BALB/c nude mice weighing 21±3 g were obtained from the Animal Laboratory of the Fourth Military Medical University. The animals were randomly divided into three groups with 21 mice in each group for tumor cell inoculation. On day 1, 100 μl cell suspension ($1x10^6$ cells) was inoculated subcutaneously into the right rear flank of the nude mice. The maximum diameter (a, mm) and vertical short diameter (b, mm) of the tumor mass were measured using a precision caliper every three days initiated on day 7 and ending on day 25. The tumor size was calculated using the following formula: V (mm^3) = a x b^2 x 0.52. The animals were sacrificed 30 days after tumor cell injection, and the tumor masses were weighted. The tumor inhibitory rate was calculated using the following formula: Tumor inhibition rate (%) = $[(V_1 - V_2)/V_1]$ x 100%, in which V_1 represents the tumor size of the OSCC-15 group and V_2 represents the tumor size of the OSCC-15+NC or OSCC-15+$p12^{CDK2\text{-}AP1}$+CD82 group. The experiment procedures involving animals and their care were conducted in conformity with the NIH guidelines (NIH Pub. no. 85-23, revised 1996) and were approved by the Animal Care and Use Committee of the Fourth Military Medical University.

Statistical analysis. The statistical analysis was conducted using SPSS19.0 software. The expression of $p12^{CDK2\text{-}AP1}$ and CD82 in oral cancer was compared using the Chi-square test. Data are presented as the means ± SD. $P<0.05$ was considered to indicate a significant difference.

Results

Prediction of $p12^{CDK2\text{-}AP1}$ and CD82 interaction. Using a multiple and pairwise kernel support vector machine, 10 proteins (including Ig α-1 chain C region, α-amylase 1 precursor, α-2 chain C region, CD82, fructose-bisphosphate aldolase A, Kruppel-like factor 6, sulfatase-modifying factor 2, inhibitor of CDK interacting with cyclin A1, insulinoma-associated protein 1 and 60S ribosomal protein L13) were predicted to have high potential in binding with $p12^{CDK2\text{-}AP1}$ (Table I). Among these proteins, tumor suppressor gene CD82 was chosen for further analysis. The sequences of $p12^{CDK2\text{-}AP1}$ and CD82 were input into the pairwise kernel support vector machine, and the two proteins were predicted as an interacting protein-protein pair (Fig. 1).

Table I. Protein interactors of $p12^{CDK2\text{-}AP1}$ identified by computational decision templates.

Swiss-Prot Protein Sequence Database	Protein
p01876	Ig α-1 chain C region
p01877	Ig α-2 chain C region
p27701	CD82 antigen
p04075	Fructose-bisphosphate aldolase A
q99612	Krueppel-like factor 6
q9by67	α-amylase 1 precursor
q8nbj7	Sulfatase-modifying factor 2
q0vd86	Inhibitor of CDK interacting with cyclin A1
q01101	Insulinoma-associated protein 1
a8k4c8	60S ribosomal protein L13

Identification of the protein-protein binding between $p12^{CDK2\text{-}AP1}$ and CD82. Co-immunoprecipitation was conducted to validate the direct binding between $p12^{CDK2\text{-}AP1}$ and CD82. For this purpose, pIRES2-EGFP-$p12^{CDK2\text{-}AP1}$ and pIRES2-EGFP-CD82 recombinant plasmids were constructed and overexpressed in the 293T cells, respectively. Western blot analysis demonstrated that $p12^{CDK2\text{-}AP1}$ and CD82 were weakly expressed in the 293T cells under baseline conditions but were gradually increased after transfection in a time-dependent manner (Fig. 2A). A statistical analysis revealed a significant upregulation of $p12^{CDK2\text{-}AP1}$ and CD82 at 72 h after transfection ($P<0.05$ compared with the control) (Fig. 2B). Additionally, bright GFP signals were detected in over 90% of the 293T cells at 72 h after transfection (Fig. 2C), indicating the successful overexpression of $p12^{CDK2\text{-}AP1}$ and CD82 in the 293T cells. A co-immunoprecipitation analysis indicated that CD82 could be specifically pulled down by the anti-$p12^{CDK2\text{-}AP1}$ antibody (Fig. 3A); conversely, $p12^{CDK2\text{-}AP1}$ could be specifically pulled down by the anti-CD82 antibody (Fig. 3B). These results demonstrated that $p12^{CDK2\text{-}AP1}$ and CD82 could be reciprocally pulled down in OSCC-15 cells, indicating a direct physical interaction between $p12^{CDK2\text{-}AP1}$ and CD82.

Overexpression of $p12^{CDK2\text{-}AP1}$ and CD82 suppresses the proliferation of OSCC-15 cells. To elucidate the potential influences of $p12^{CDK2\text{-}AP1}$ and CD82 on the growth, proliferation and survival of human oral squamous cell carcinoma cells, $p12^{CDK2\text{-}AP1}$ and CD82 were overexpressed in the OSCC-15 cells via plasmid transfection. At 72 h after transfection, over 90% of the OSCC-15 cells expressed GFP signals (Fig. 4A).

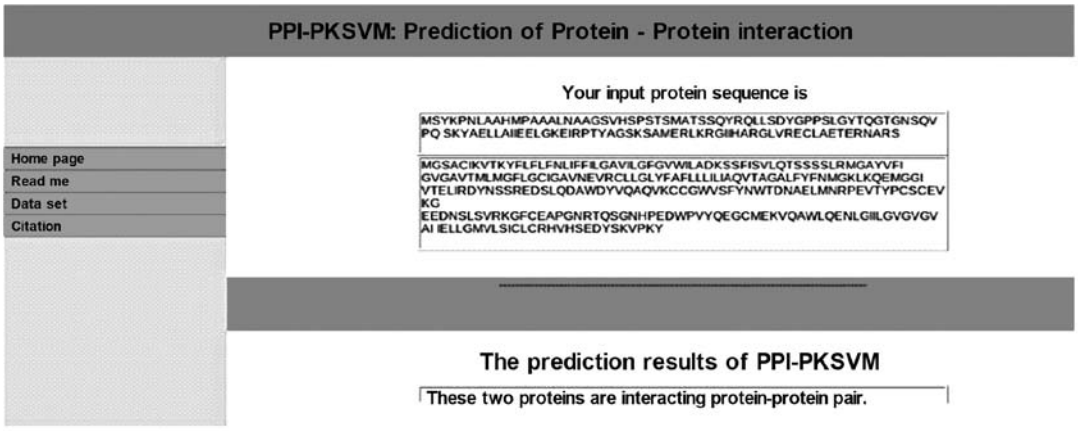

Figure 1. Protein-protein interaction between p12$^{CDK2-AP1}$ and CD82. The protein-protein interaction was determined by a decision template-based multiple and pairwise kernel support vector machine. The sequences of p12$^{CDK2-AP1}$ and CD82 were input into the pairwise kernel support vector machine; the prediction results are shown.

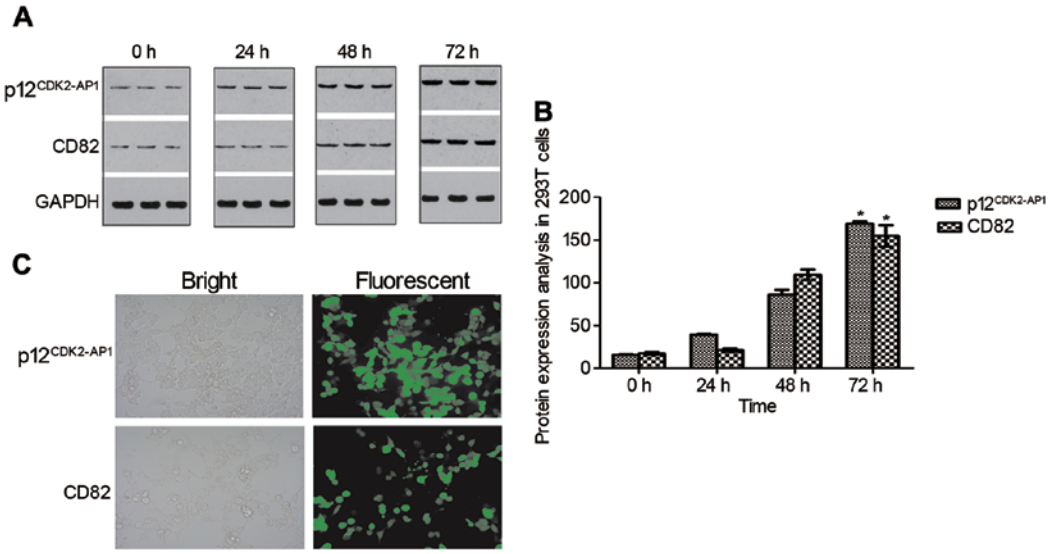

Figure 2. Overexpression of p12$^{CDK2-AP1}$ and CD82 in 293T cells. Cells were transfected with either pIRES2-EGFP-p12$^{CDK2-AP1}$ or pIRES2-EGFP-CD82 recombinant plasmid. (A) At 0, 24, 48 and 72 h after transfection, the total proteins were collected, and the protein levels of p12$^{CDK2-AP1}$ and CD82 were examined via western blotting. GAPDH was used as a loading control. (B) The protein levels of p12$^{CDK2-AP1}$ and CD82 were normalized via GAPDH, and the relative protein level was calculated. Data are presented as the means ± SD. n=3. *P<0.05 compared with 0 h. (C) The GFP signal in the transfected 293T cells at 72 h was examined under a fluorescence microscope. Original magnification, x100.

The MTT analysis showed that transfection with the negative control (NC) vector did not significantly affect cell proliferation (P>0.05 compared with the control) (Fig. 4B). The overexpression of either p12$^{CDK2-AP1}$ or CD82 significantly suppressed cell proliferation at 72 and 96 h after transfection (P<0.05 compared with the NC). Notably, the combined transfection of pIRES2-EGFP-p12$^{CDK2-AP1}$ and pIRES2-EGFP-CD82 yielded an enhanced growth inhibition (inhibition rate of 1.68%) compared with single plasmid transfection (P<0.05 compared with the NC), suggesting that p12$^{CDK2-AP1}$ and CD82 may synergistically inhibit the proliferation of OSCC-15 cells. These data suggest that the overexpression of p12$^{CDK2-AP1}$ and CD82 inhibits the proliferation of OSCC-15 cells.

Overexpression of p12$^{CDK2-AP1}$and CD82 inhibits the invasion of OSCC-15 cells. We next investigated the effects of p12$^{CDK2-AP1}$ and CD82 overexpression on the invasion capability of OSCC-15 cells *in vitro* using a Transwell system. There were no significant differences in the number of invaded cells between the non-transfected control and the cells transfected with the NC plasmid (P>0.05, Fig. 5). However, the overexpression of either p12$^{CDK2-AP1}$ or CD82 significantly inhibited cell invasion (P<0.05 compared with the NC). Additionally, combined transfection of pIRES2-EGFP-p12$^{CDK2-AP1}$ and pIRES2-EGFP-CD82 led to a more efficient inhibition of cell invasion compared with single plasmid transfection (P<0.05; Fig. 5), indicating a synergistic inhibitory effect by p12$^{CDK2-AP1}$ and CD82 on cell invasion. This evidence indicates that the overexpression of p12$^{CDK2-AP1}$ and CD82 can suppress the *in vitro* invasion capability of OSCC-15 cells.

Overexpression of p12$^{CDK2-AP1}$ and CD82 induces apoptosis in OSCC-15 cells. To assess the potential role of p12$^{CDK2-AP1}$ and CD82 on cell apoptosis, OSCC-15 cells transfected with

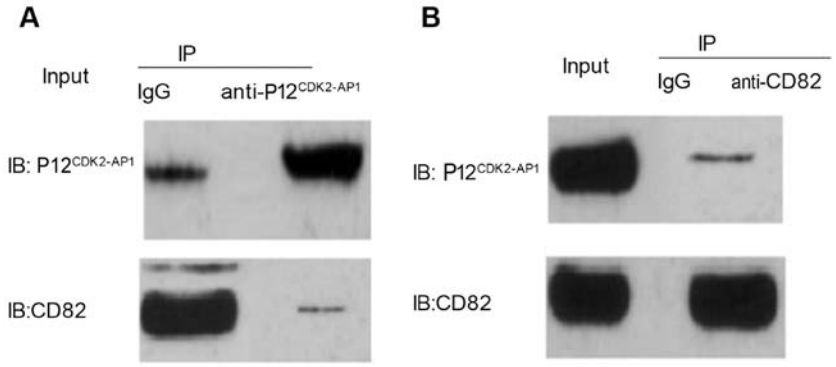

Figure 3. Interaction between $p12^{CDK2\text{-}AP1}$ and CD82 as determined via co-immunoprecipitation. (A) Western blot analysis of aliquots from cell lysates (input) from OSCC-15 cells transfected with pIRES2-EGFP-$p12^{CDK2\text{-}AP1}$ and pIRES2-EGFP-CD82 recombinant plasmids of aliquots from supernatants of the Co-IP (IgG, control condition, or with antibody against $p12^{CDK2\text{-}AP1}$). (B) Western blot analysis of aliquots from cell lysates (input) from OSCC-15 cells transfected with pIRES2-EGFP-$p12^{CDK2\text{-}AP1}$ and pIRES2-EGFP-CD82 recombinant plasmids of aliquots from supernatants of the Co-IP (IgG, control condition, or with antibody against CD82).

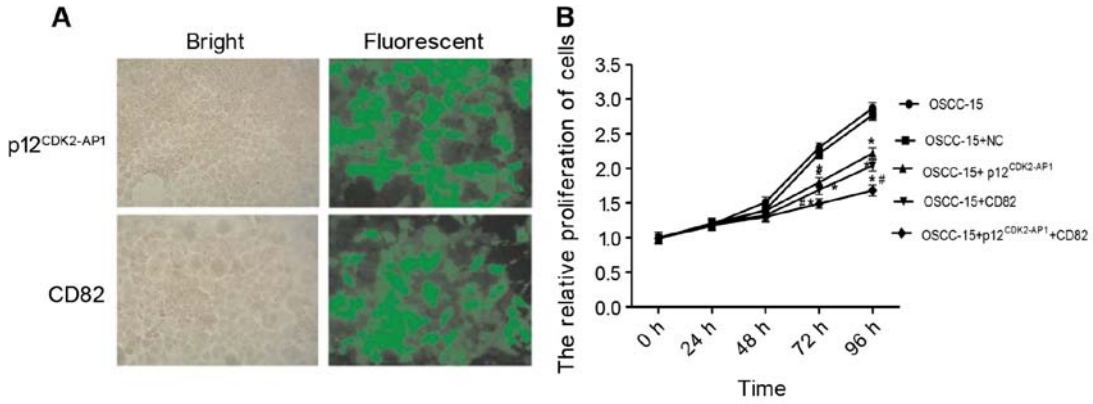

Figure 4. Overexpression of $p12^{CDK2\text{-}AP1}$ and CD82 suppresses the proliferation of OSCC-15 cells. (A) OSCC-15 cells were transfected with either pIRES2-EGFP-$p12^{CDK2\text{-}AP1}$ or pIRES2-EGFP-CD82 recombinant plasmid. At 72 h after transfection, the GFP signal was examined under a fluorescence microscope. Original magnification, x100. (B) OSCC-15 cells were transfected with pIRES2-EGFP (NC), pIRES2-EGFP-$p12^{CDK2\text{-}AP1}$, pIRES2-EGFP-CD82 or pIRES2-EGFP-$p12^{CDK2\text{-}AP1}$ plus pIRES2-EGFP-CD82 plasmids. Non-transfected cells were included as a control. The cell proliferation ability was determined via MTT assay at 0, 24, 48, 72, and 96 h after transfection. The relative proliferation rates of the cells were calculated from 3 independent experiments and are presented as the means ± SD. *P<0.05 vs. OSCC-15 + NC group; $^{\#}$P<0.05 vs. OSCC-15+$p12^{CDK2\text{-}AP1}$ or OSCC-15+CD82 group.

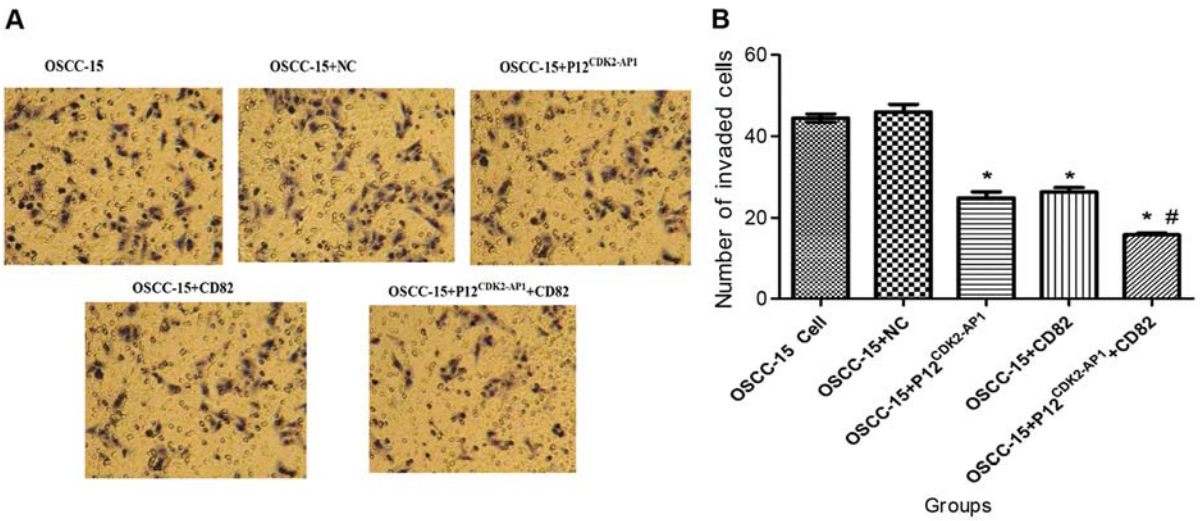

Figure 5. Overexpression of $p12^{CDK2\text{-}AP1}$ and CD82 suppresses the invasion of OSCC-15 cells. OSCC-15 cells were transfected with pIRES2-EGFP (NC), pIRES2-EGFP-$p12^{CDK2\text{-}AP1}$, pIRES2-EGFP-CD82 or pIRES2-EGFP-$p12^{CDK2\text{-}AP1}$ plus pIRES2-EGFP-CD82 plasmids. Non-transfected cells were included as a control. Cell invasion capability was determined using a Transwell system. (A) Representative images of invaded cells detected under a light microscope. Original magnification, x100. (B) The number of invaded cells was counted. The average number of invaded cells was calculated from three independent experiments and is presented as the means ± SD. *P<0.05 vs. OSCC-15+NC group; $^{\#}$P<0.05 vs. OSCC-15+$p12^{CDK2\text{-}AP1}$ or OSCC-15+CD82 group.

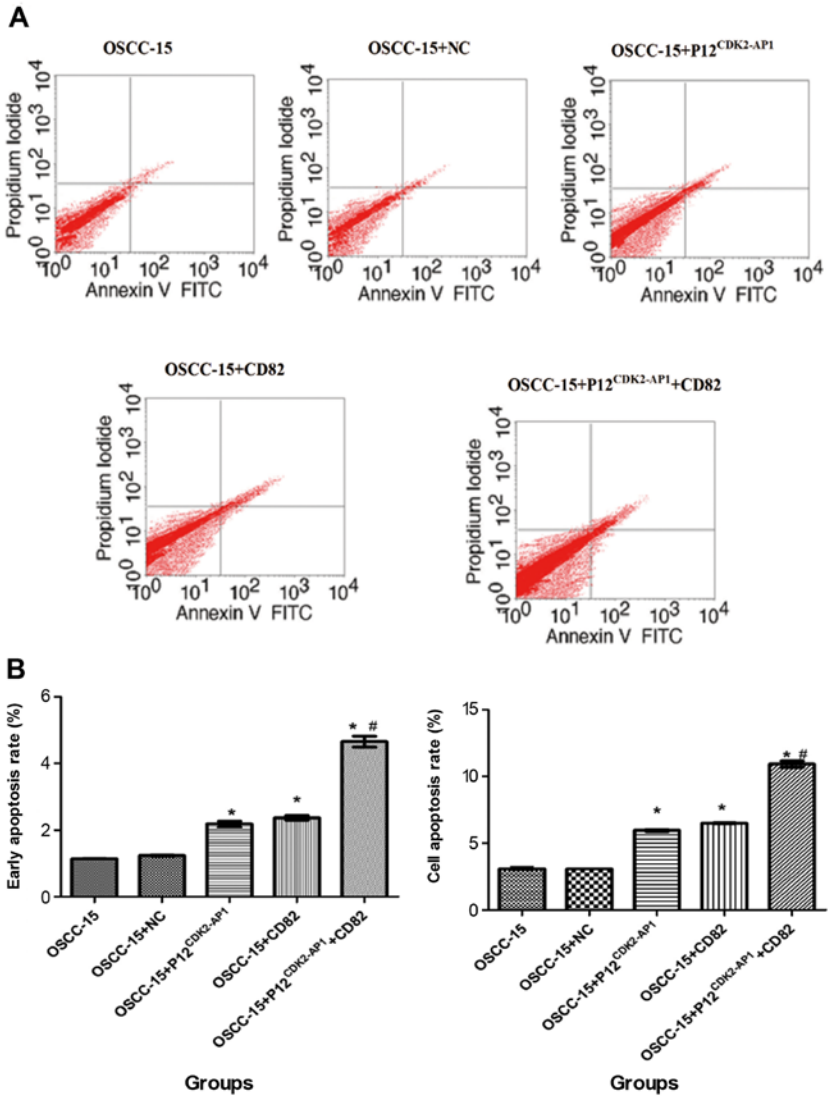

Figure 6. Overexpression of p12$^{CDK2-AP1}$ and CD82 promotes the apoptosis of OSCC-15 cells. OSCC-15 cells were transfected with pIRES2-EGFP (NC), pIRES2-EGFP-p12$^{CDK2-AP1}$, pIRES2-EGFP-CD82 or pIRES2-EGFP-p12$^{CDK2-AP1}$ plus pIRES2-EGFP-CD82 plasmids. Non-transfected cells were included as a control. The cells were co-stained with Annexin V-FITC and PI followed by flow cytometric analysis. (A) Representative flow cytometric analysis data. (B) The percentage of early apoptotic and apoptotic cells was calculated from three independent experiments and is presented as the means ± SD. *P<0.05 vs. OSCC-15+NC group; $^{\#}$P<0.05 vs. OSCC-15+p12$^{CDK2-AP1}$ or OSCC-15+CD82 group.

NC, pIRES2-EGFP-p12$^{CDK2-AP1}$ and pIRES2-EGFP-CD82 alone or in combination were probed with Annexin V-FITC and PI, followed by flow cytometry analysis. The transfection of NC did not significantly influence cell apoptosis compared with that in the non-transfected control cells (P>0.05) (Fig. 6). However, the overexpression of p12$^{CDK2-AP1}$ or CD82 significantly increased the percentage of both the early and overall apoptotic cells (P<0.05 compared with the NC). Moreover, the combined transfection of both plasmids was significantly more efficient in promoting cell apoptosis compared with single plasmid transfection (P<0.01), suggesting a synergistic effect for p12$^{CDK2-AP1}$ and CD82 on cell apoptosis. These data indicate that the overexpression of p12$^{CDK2-AP1}$ and CD82 induces apoptosis in OSCC-15 cells.

Overexpression of p12$^{CDK2-AP1}$ and CD82 inhibits the in vivo growth of OSCC-15 cells in tumor mouse xenografts. Finally, we investigated the combined overexpression of p12$^{CDK2-AP1}$ and CD82 on the growth of OSCC-15 cells *in vivo*. To this end, OSCC-15 cells transfected with NC or those transfected with pIRES2-EGFP-p12$^{CDK2-AP1}$ plus pIRES2-EGFP-CD82 were subcutaneously injected into nude mice. Twenty-five days after inoculation, no significant differences were detected regarding the size of the tumor mass between the non-transfected OSCC-15 cell group and the OSCC-15 cells transfected with NC (Fig. 7A). However, the combined overexpression of p12$^{CDK2-AP1}$ and CD82 substantially reduced the tumor size. Additionally, the combined overexpression of p12$^{CDK2-AP1}$ and CD82 significantly reduced the tumor volume and yielded a significant tumor inhibitory rate at 25 days after inoculation compared with the NC group (Fig. 7B and C; P<0.05). These findings suggest that the combined overexpression of p12$^{CDK2-AP1}$ and CD82 inhibits the growth of OSCC-15 cells in tumor mouse xenografts.

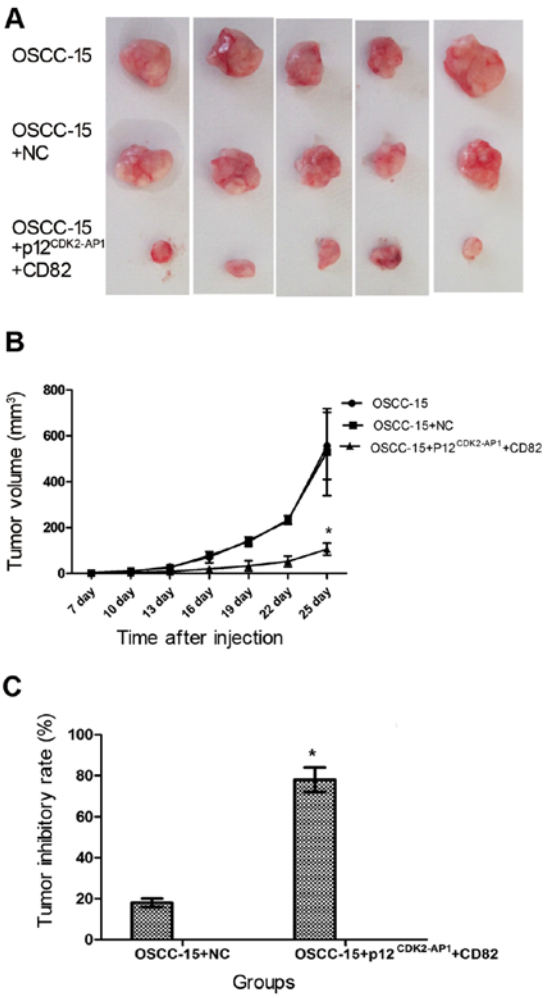

Figure 7. Overexpression of $p12^{CDK2\text{-}AP1}$and CD82 inhibits the *in vivo* growth of OSCC-15 cells in nude mice. OSCC-15 cells were transfected with pIRES2-EGFP (NC) or pIRES2-EGFP-$p12^{CDK2\text{-}AP1}$ plus pIRES2-EGFP-CD82 plasmids. Non-transfected cells were included as a control. The cells were inoculated subcutaneously into the right rear flank of nude mice, and the tumor volume (mm^3) was calculated every three days following inoculation. Twenty-five days after inoculation, the mice were sacrificed and the tumors were dissociated. (A) Gross morphology of tumor mass. (B) Tumor growth curve in nude mice. (C) Tumor inhibitory rate in nude mice. $^*P<0.05$ vs. OSCC-15+NC group. n=3 for each time-point. n=7 for each group.

Discussion

In this study, using a multiple and pairwise kernel support vector machine, 10 proteins were predicted to have high binding potential with $p12^{CDK2\text{-}AP1}$. Among these proteins, CD82 was further verified to interact with $p12^{CDK2\text{-}AP1}$ via co-immunoprecipitation. Moreover, the overexpression of either $p12^{CDK2\text{-}AP1}$ or CD82 significantly inhibited proliferation and invasion but induced apoptosis in the OSCC-15 cells. However, the combined overexpression of $p12^{CDK2\text{-}AP1}$ and CD82 was significantly more efficient in promoting cell apoptosis and inhibiting proliferation and invasion compared with single overexpression. Importantly, the combined overexpression of $p12^{CDK2\text{-}AP1}$ and CD82 inhibited the *in vivo* growth of OSCC-15 cells in tumor mouse xenografts. Our data suggest that $p12^{CDK2\text{-}AP1}$ and CD82 may synergistically inhibit the growth of OSCC-15 cells.

The dysregulated cell cycle control system is a crucial process during oral carcinogenesis (15). Cyclin-dependent kinases (CDKs) are widely accepted to control cell cycle progression. $p12^{CDK2\text{-}AP1}$ has been found to regulate cell cycle progression and cell proliferation by negatively mediating the kinase activities of CDK2 (16). A growing body of evidence supports the notion that $p12^{CDK2\text{-}AP1}$ inhibits the progression of several human cancers, such as breast cancer (17), gastric cancer (18), and esophageal squamous cell carcinoma (19). The silencing of $p12^{CDK2\text{-}AP1}$ expression was reported to accelerate the proliferation of human skin keratinocyte (HaCaT) cells (20), whereas the upregulation of $p12^{CDK2\text{-}AP1}$ expression led to the reduced proliferation and invasion of HaCaT cells (21). Consistent with these findings, in the present study, the overexpression of $p12^{CDK2\text{-}AP1}$ in OSCC-15 cells significantly suppressed cell proliferation and invasion and promoted cell apoptosis, indicating the inhibitory role of $p12^{CDK2\text{-}AP1}$ in OSCC.

Increasing evidence indicates that $p12^{CDK2\text{-}AP1}$ interacts with other proteins such as DNA polymerase α/primase (9) and the homologous protein p14 (10). Using a yeast two-hybrid system, our previous study also identified a novel unnamed protein product (UPP) that interacts with $p12^{CDK2\text{-}AP1}$ and inhibits the proliferation of 293T and HeLa cells (22). Using computational approaches, CD82 was predicted to be an interacting protein of $p12^{CDK2\text{-}AP1}$, which was further confirmed via Co-IP analysis. CD82, also known as KAI1, was originally identified in human prostate carcinoma and mapped to human chromosome 11p11.2 (23). CD82 has been shown to be a metastasis suppressor in many types of human cancers, including prostate (24), bladder (25), breast (26), colon (27), pancreas (28), and lung (29,30) cancers. Additionally, the mutation or downregulation of CD82 protein has been detected in esophageal (31,32) and oral (33-35) cancers. In non-small cell lung carcinoma h1299 cells, CD82 was found to negatively regulate cell motility and migration (36,37). However, the regulatory role of CD82 in OSCC cells is not yet fully understood. In this study, we found that overexpression of CD82 yielded an inhibitory effect on OSCC-15 cells similar to that of $p12^{CDK2\text{-}AP1}$, implying that CD82 may also act as a tumor suppressor in OSCC.

More importantly, our results revealed that when $p12^{CDK2\text{-}AP1}$ and CD82 were co-expressed in OSCC-15 cells, a synergistic tumor inhibition was detected both *in vitro* and *in vivo*. Compared with single plasmid transfection, the combined transfection of $p12^{CDK2\text{-}AP1}$ with CD82 demonstrated more efficiency in suppressing cell growth and invasion and promoting cell apoptosis. Moreover, the simultaneous overexpression of $p12^{CDK2\text{-}AP1}$ and CD82 significantly inhibited the growth of OSCC-15 cells in tumor mouse xenografts. These data indicate that $p12^{CDK2\text{-}AP1}$ and CD82 serve as tumor suppressors and may act synergistically in suppressing the tumorigenesis of OSCC.

In summary, our results demonstrated that $p12^{CDK2\text{-}AP1}$ interacted with CD82 and negatively regulated the growth and survival of OSCC-15 cells. Moreover, our findings suggest that targeting the interaction between $p12^{CDK2\text{-}AP1}$ and CD82 may represent a potential strategy for the development of treatment strategies for oral cancers. Future studies will be needed to explore the molecular mechanisms in modulating the $p12^{CDK2\text{-}AP1}$-CD82 interaction.

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