Journal of Cell Communication and Signaling (2022) 16:567–578
https://doi.org/10.1007/s12079-022-00670-6

RESEARCH ARTICLE

# ACK1 upregulated the proliferation of head and neck squamous cell carcinoma cells by promoting p27 phosphorylation and degradation

**Hsuan-Hsiang Peng[1] · Hao-Chin Yang[1] · Darius Rupa[1] · Chun-Han Yen[1] · Ya-Wen Chiu[1] · Wei-Jia Yang[2] · Fuh-Jinn Luo[2] · Ta-Chun Yuan[1]**

Received: 31 May 2021 / Accepted: 2 February 2022 / Published online: 5 March 2022


## Abstract

Head and neck squamous cell carcinoma (HNSCC) is a malignancy with a worldwide distribution. Although intensive studies have been made, the underlying oncogenic mechanism of HNSCC requires further investigation. In this study, we examined the oncogenic role of activated Cdc42-associated kinase 1 (ACK1), an oncogenic tyrosine kinase, in regulating the proliferation of HNSCC cells and its underlying molecular mechanism. Results from immunohistochemical studies revealed that ACK1 was highly expressed in HNSCC tumors, with 77% (77/100) of tumors showing a high ACK1 immunoreactivity compared to 40% (8/20) of normal mucosa. Knockdown of ACK1 expression in HNSCC cells resulted in elevated p27 expression, reduced cell proliferation, and G1-phase cell cycle arrest. Rescue of ACK1 expression in the ACK1-knockdown cells suppressed p27 expression and restored cell proliferation. Compared to ACK1-knockdown cells, ACK1-rescued cells exhibited a restored p27 expression after MG132 treatment and showed an elevated level of ubiquitinated p27. Our data further showed that knockdown of ubiquitin ligase Skp2 resulted in elevated p27 expression. Importantly, the expression of p27(WT), p27(Y74F), or p27(Y89F) in ACK1-overexpressed 293T cells or ACK1-rescued SAS cells showed higher levels of tyrosyl-phosphorylated p27 and interaction with ACK1 or Skp2. However, the expression of p27(Y88F) mutant exhibited a relatively low phosphorylation level and barely bound with ACK1 or Skp2, showing a basal interaction as the control cells. These results suggested that ACK1 is highly expressed in HNSCC tumors and functions to promote cell proliferation by the phosphorylation and degradation of p27 in the Skp2-mediated mechanism.

**Keywords** ACK1 · HNSCC · p27 · Skp2 · Tyrosine phosphorylation

## Introduction

Head and neck squamous cell carcinoma (HNSCC) is a malignant disease in the oral cavity, pharynx, and larynx, affecting over 700,000 patients per year worldwide (Bray et al. 2018). Although intensive studies have been made in the diagnosis and treatment, the overall 5-year survival rates of patients are approximately 60% (Marur and Forastiere 2016). One of the reasons for mortality is the lack of effective treatment. Accumulated evidence suggested the predominant role of receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR), in HNSCC malignancy, which is considered as the optimal therapeutic target for treating HNSCC. However, clinical trials of targeting EGFR in HNSCC with specific inhibitors failed to obtain satisfactory results. For example, the response rate of patients to cetuximab, an EGFR-targeted monoclonal antibody, is only 13% in the monotherapy and 36% in combination with chemotherapy (Vermorken et al. 2007, 2008). These results revealed that other molecules or signaling pathways, in addition to receptor tyrosine kinases, are critical for promoting HNSCC malignancy.

ACK1, also called TNK2, is a structurally unique non-receptor tyrosine (Y) kinase that is linked to cellular transformation. Functionally, ACK1 integrates signals from

Hsuan-Hsiang Peng and Hao-Chin Yang contributed equally to this work.

✉ Ta-Chun Yuan
yuan415@gms.ndhu.edu.tw

[1] Department of Life Science, National Dong Hwa University, No. 1, Sec. 2, Da Hsueh Rd., Shoufeng, Hualien 974301, Taiwan, Republic of China

[2] Department of Pathology, Mennonite Christian Hospital, Hualien 970, Taiwan, Republic of China

ligand-activated receptor tyrosine kinases and activates intracellular signaling cascades to regulate cellular functions (Galisteo et al. 2006; Pao-Chun et al. 2009; Mahajan and Mahajan 2015). Due to gene amplification or missense mutations, aberrant activation of ACK1 is commonly found in human cancers and is associated with transformed phenotypes (van der Horst et al. 2005; Prieto-Echague et al. 2010; Mahajan and Mahajan 2015). Aberrantly expressed or activated ACK1 can modulate the activities of multiple proteins by tyrosine phosphorylation. ACK1 has been proved to phosphorylate AKT at Y176, promoting AKT activation and leading to cell proliferation and survival (Mahajan et al. 2010b). ACK1 can interact with the androgen receptor (AR) in prostate cancer cells and phosphorylate it at Y267 and Y363, facilitating androgen-independent AR activation (Mahajan et al. 2007). Alternatively, ACK1 phosphorylates histone H4 at Y88 at the AR transcription start site, which causes the recruitment of the WDR5/MLL2 complex and enhances AR transcription (Mahajan et al. 2017). ACK1 also phosphorylates estrogen receptor co-activator KDM3A at Y1114, promoting the tamoxifen-resistance phenotype in breast cancer (Mahajan et al. 2014). While ACK1 expression and function have been intensively studied in different cancer types, the oncogenic role of ACK1 in HNSCC cells and the possible regulatory mechanism is still unclear.

$p27^{Kip1}$ (p27) is a member of the kinase inhibitor protein family, which plays an important role in controlling cell cycle progression (Hnit et al. 2015). By direct binding, p27 inhibits cyclin E/A-Cdk2 complex and causes cell cycle G1 arrest (Okuyama et al. 2021). According to the literature, p27 mutation in human cancer is rare but reduced p27 expression is closely associated with tumor size, disease progression, and poor prognosis (Migita et al. 2002). Indeed, the downregulation of p27 was found in HNSCC tumors with a positive rate of p27 varying from 10 to 56% in eight independent studies (de Almeida et al. 2015). Importantly, the downregulation of p27 in tumors is primarily mediated by ubiquitin-dependent proteolysis mechanisms and requires p27 phosphorylation (Okuyama et al. 2021). The p27 can be phosphorylated at multiple serine (S) and threonine (T) residues, including S10, T157, T187, and T198, which are critical for its cytoplasmic localization or proteasomal degradation (Hnit et al. 2015). A line of evidence further demonstrated that the tyrosine phosphorylation of p27 is also crucial for its proteasomal degradation and cyclin-dependent kinase (Cdk) activation. Among 198 amino acids in p27, only three tyrosine residues, i.e., Y74, Y88, and Y89, are found and all are located in the Cdk binding domain. So far, several non-receptor tyrosine kinases have been reported to phosphorylate p27, including c-Src, Yes, Lyn, Brk, JAK2, and Abl (Jakel et al. 2012; Patel et al. 2015). Whether ACK1 can phosphorylate p27 and lead to its proteasomal degradation is currently unknown.

In this study, we found that ACK1 was highly expressed in HNSCC tumors. The expression of ACK1 was closely associated with the decreased p27 expression and the increased proliferation in HNSCC cells. Our data further showed that ACK1 enhanced the ubiquitination and proteasomal degradation of p27. Importantly, ACK1 could bind to p27 and phosphorylate it at Y88, which is critical for recruiting Skp2 to p27. Together, our data suggested the oncogenic role of ACK1 in modulating HNSCC cell proliferation by promoting p27 phosphorylation and degradation. Thus, ACK1 could potentially serve as a therapeutic target for HNSCC.

## Materials and methods

### Antibodies and chemicals

The primary antibodies against ACK1 (sc-28336), COP1 (sc-166799), Skp2 (sc-74477), KPC1 (sc-101122), ubiquitin (sc-8017), β-actin (sc-69879) were obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). Antibodies against p21 (ab109520), p27 (ab32034), or cyclin D1 (ab134175) were purchased from Abcam (Cambridge, UK). The anti-phosphotyrosine (pY) antibodies (AP0905) were from ABclonal (Woburn, MA, USA), and the anti-Flag M2 antibodies (F1804) were from Sigma-Aldrich (Burlington, MA, USA). HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology. Proteasome inhibitor MG132 (C2211), (R)-9bMS (SML2073), cycloheximide (C7698), and other chemicals were obtained from Sigma-Aldrich (Burlington, MA, USA).

### Cell culture

293T, OECM-1, HSC-3, or SAS cells were purchased from the JCRB Cell Bank (Osaka, Japan) or kindly provided by Dr. Kuo-Wei Chang (National Yang-Ming University, Taipei, Taiwan). OECM-1 cells were cultured in RPMI 1640 medium. 293T, HSC-3, and SAS cells were cultured in the DMEM medium. Each medium was supplemented with 5% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cell culture media and supplements were purchased from Invitrogen.

### cDNA expression vector construction and site-directed mutagenesis

The full-length mouse ACK1 cDNA carried by the pmCherry-C1 vector was a gift from Christien Merrifield (Addgene plasmid # 27684). The cDNA was amplified using oligonucleotide primers: (forward): 5'-GCGATTTAAATCCCCGTAATGCAGAAGAAGA-3' (SwaI site

is underlined), and (Reverse):5'-GCGGCGGCCGCGAA ATTTGTGATGCTATTGC-3' (NotI site is underlined). The PCR products were digested by SwaI/NotI and subsequently ligated with the SwaI/NotI-cut pCDH-CMV-MCS-EF1-puro (pCDH) lentiviral vector (System Biosciences). The ligated products were designated as pCDH-ACK1(WT). For the construction of p27-containing plasmid, we have purchased the pCMV-SPORT6 expression vector carrying the wild-type human p27 cDNA from Bioresource Collection and Research Centre (BCRC, Hsinchu, Taiwan). The PCR reaction (94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min; 30 cycles) were conducted using the paired primers: (forward) 5'-GGGTCTAGAATGGACTACAAAGACGAT GACGACAAGTCAAACGTGCGAGTGTCT-3' (XbaI site is underlined), and (reverse): 5'-GGGGGATCCTTACGT TTGACGTCTTCTGAG-3' (BamHI site is underlined). The PCR products were then digested by XbaI/BamHI and subsequently ligated with the XbaI/BamHI-cut pCDH vector. The ligated products were designated as pCDH-Flag-p27(WT). To further prepare Y74F, Y88F, and Y89F mutant constructs of p27, the pCMV-SPORT6-p27 plasmid was served as a template and the following oligonucleotides were used as the primers: The cDNA was amplified using oligonucleotide primers: The Y74F forward: 5'-GAGGGC AAG**TTC**GAGTGGCAAGAGGTGGAGAAGGGCAGC TTGCCC-3', and reverse: 5'-TTGCCACTC**GAA**CTTGCC CTCTAGGGGTTTGTGATTCTGAAAATC-3'. The Y88F forward: 5'-CCCGAGTTC**TTC**TACAGACCCCCGCGG CCCCCCAAAGCTGC-

CTGC-3' and reverse: 5'-GGGTCTGTA**GAA**GAACTC GGGCAAGCTGCCCTTCTCCAC-

CTCTTG-3'. The Y89F forward: 5'-GAGTTCTAC**TTC** AGACCCCCGCGGCCCCCCAA-

AGCTGCCTGC-3' and reverse: 5'-CGGGGGTCT**GAA** GTAGAACTCGGGCAAGCTG-

CCCTTCTCCACCTC-3' (mutated nucleotides are underlined). The mutagenized PCR fragments have been sequenced to confirm the mutations and then amplified using the following primers: The forward: '-GGG**TCTAGA***ATG GACTACAAAGACGATGACGACA-*

*AG*TCAAACGTGCGAGTGTCT-3' (XbaI site is underlined and the Flag sequence is showed in *italics*) and reverse: 5'-GGG**GGATCC**TTACGTTTGACGTCTTCTGAG-3' (BamHI site is underlined). Subsequently, the mutagenized PCR fragments were digested by XbaI/BamHI and ligated with the enzyme-cut pCDH lentiviral vector. The ligated products were designated as pCDH-Flag-p27(Y74F), pCDH-Flag-p27(Y88F), and pCDH-Flag-p27(Y89F).

## Lentiviral transduction

For gene knockdown experiments, lentiviral vectors carrying shRNA targeting the coding sequence (i.e, shACK1-1 or shACK1-2) or the 3'-UTR (i.e., shACK1) of human *TNK2* gene were obtained from the RNAi Core at Academic Sicina (Taipei, Taiwan). Lentivirus transfection was carried out according to the protocol described previously (Hsiao et al. 2016). Briefly, 293T cells were co-transfected with the lentiviral plasmids plus packaging plasmids, using a PolyJet Transfection Reagent from SignaGen Lab (Frederick, MD, USA). The control cells were co-transfected with the control vector plus packaging plasmids. After 48 h of incubation, the culture medium containing lentiviruses was harvested. For infection, cells grown in 6-well plates were incubated with viruses in the presence of 8 μg/mL of polybrene overnight at 37 °C. After refreshing with the media, the infected cells were grown for 48 h and may undergo the puromycin selection for an additional 24 or 48 h.

## Cell proliferation assay

A density of $0.7\text{-}1 \times 10^5$ cells/well was plated on 24-well plates. After 48 h of incubation, one set of attached cells was harvested and counted as day 0, while the remaining cells were fed with fresh medium, and the cell number was counted on day 3. Alternatively, cell counting kit-8 (Enzo, New York, USA) was used to analyze the effect of (R)-9bMS on cell proliferation. Briefly, a density of $5 \times 10^3$ cells/well in 100 μL was seeding in a 96-well plate, and 10 μL CCK-8 solution was added to each well. After incubating the plate for 1-4 h, the absorbance at 450 nm was measured using a microplate reader.

## Cell cycle analysis

Cells were harvested and fixed in ice-cold 70% ethanol. After washing with PBS, the cell pellets were stained with 1 mL of propidium iodide (PI) staining solution containing 0.1% Triton X-100, 20 μg/mL PI, and 200 μg/mL RNase for 30 min. Acquisition and analysis were performed by Cytomics FC500 flow cytometry (Beckman Coulter, Brea, CA, USA) with excitation at 488 nm.

## Immunoprecipitation and immunoblotting

For co-immunoprecipitation, 0.7-1 mg of supernatant protein was incubated with Ab-conjugated protein A/G-Sepharose complexes overnight at 4 °C. The immunocomplexes were spun at 10000xg for 1 min, washed three times with ice-cold lysis buffer and suspended in an SDS-PAGE sample buffer. The immunoblot analyses were performed as previously described (Chang et al. 2014). The relative level of p27 protein was semi-quantified by densitometric analysis using ImageJ from National Institutes of Health (Bethesda, MA, USA).

### Tissue microarray and immunohistochemistry

Tissue arrays of head and neck regions (OR601b and HN803d) containing normal and malignant samples were purchased from US Biomax (Rockville, MD, USA). The cores on these samples were 1.5 mm in diameter. Immunohistochemistry was performed using a Bond Max Automated Immunostainer and reagents from Leica Biosystems (Wetzlar, Germany). Briefly, the slides were dewaxed in Bond Dewax solution and hydrated in Bond Wash solution. Antigen retrieval was performed at alkaline pH using Epitope Retrieval 2 solution for 20 min at 100℃. The slides were subsequently incubated with anti-ACK1 antibodies (ab185726) from Abcam (Cambridge, UK) at 1:100 dilution for 30 min. Detection of the antibodies was performed using the Bond Polymer Refine Detection (DS9800, Leica Biosystems) according to the manufacturer's protocol. The slides were counterstained with hematoxylin for 2.5 min. The expression of ACK1 was evaluated independently by two experienced pathologists, Dr. F-J Luo (30 years of experience) and Dr. W-J Yang (6 years of experience). We adopted the examination method was adopted from Okuyama K et al. (Okuyama et al. 2019). Briefly, the total immunostaining score of ACK1 expression was ranged from 0 to 7, which was the sum of the positive area score (0, none; 1, <10%; 2, 10–50%; 3, 50–80%; 4, >80%) and the intensity score (0, no staining; 1, weak; 2, moderate; 3, strong) in each tumor section. The total score of ACK1 $\leq 3$ was defined as the low expression, while the score $\geq 4$ was considered the high expression.

### Statistical analysis

Significance for group comparisons was assessed by Student's two-tailed t-test or one-way ANOVA followed by Dunnett's post hoc comparison. The Fisher's exact test was used for comparison between categorical variables. A $P$ value <0.05 was considered significant. All statistical analyses were performed using Prism 6 (GraphPad Software, San Diego, CA, USA).

## Results

### The expression of ACK1 was elevated in HNSCC tissues

To examine the oncogenic role of ACK1 in HNSCC cells, we first examined the clinical significance of ACK1 expression in HNSCC specimens by immunohistochemical analyses. Our data clearly showed that ACK1 was highly expressed in HNSCC tumors compared to normal tissues. A high level of ACK1 immunostaining was found in 77% (77/100) of malignant tissues in comparison with 40% (8/20) of normal epithelia ($p$=0.0021, Table 1). The representative images of normal epithelia (Fig. 1a) and HNSCC tissues (Fig. 1b-d) displayed differential ACK1 immunoreactivities. Table 1 summarizes the intensity data of ACK1 immunostaining in arrayed tissues, including 100 HNSCC tumors. Among them, 77% (77/100) of tumors showed a high ACK1 immunoreactivity, whereas 23 cases (23/100; 23%) displayed low immunoreactivity. In addition, there was no significant association between ACK1 expression and tumor size, nodal stage, or tumor grade. These results suggested that ACK1 was highly expressed in HNSCC tumors.

### Depleting of ACK1 expression caused elevated p27 expression, reduced cell proliferation, or G1-phase arrest in HNSCC cells

To examine whether ACK1 is involved in regulating the proliferation of HNSCC cells and the possible molecular mechanism, we analyzed the expression levels of three cell cycle regulatory proteins, i.e., p21, p27, and cyclin D1, in ACK1-knockdown HNSCC cells. As shown in Fig. 2a, the ACK1-knockdown OECM-1 cells exhibited increased levels of p27, approximately 2.5-3.4 folds higher than that of the shCt control cells. Decreased ACK1 levels were also correlated with reduced expression of p21 but did not affect cyclin D1. Again, elevated p27 expression was also found in ACK1-knockdown HSC-3 or SAS cells. However, the changes of p21 and cyclin D1 levels in those cells were

**Table 1** Association of ACK1 expression with clinicopathological parameters in HNSCC specimens

| Tissues | n (100%) | Low (%) | High (%) | $p$ value |
|---|---|---|---|---|
| Normal epithelium | 20 | 12 (60.0) | 8 (40.0) | 0.0021* |
| HNSCC | 100 | 23 (23.0) | 77 (77.0) | |
| *Age (mean)* | | | | |
| ≤ 56 | 52 | 12 (23.1) | 40 (76.9) | 1.0 |
| >56 | 48 | 11 (22.9) | 37 (77.1) | |
| *Gender* | | | | |
| Male | 71 | 15 (21.1) | 56 (78.9) | 0.601 |
| Female | 29 | 8 (27.6) | 21 (72.4) | |
| *Tumor size* | | | | |
| T1+T2 | 74 | 17 (23.0) | 57 (77.0) | 1.0 |
| T3+T4 | 26 | 6 (23.1) | 20 (76.9) | |
| *Nodal stage* | | | | |
| N=0 | 79 | 18 (22.8) | 61 (77.2) | 1.0 |
| N>0 | 21 | 5 (23.8) | 16 (76.2) | |
| *Tumor grade* | | | | |
| I | 50 | 14 (26.0) | 36 (74.0) | 0.342 |
| II+III | 50 | 9 (9.1) | 41 (81.8) | |

*$p$ value was identified by Fisher's exact test

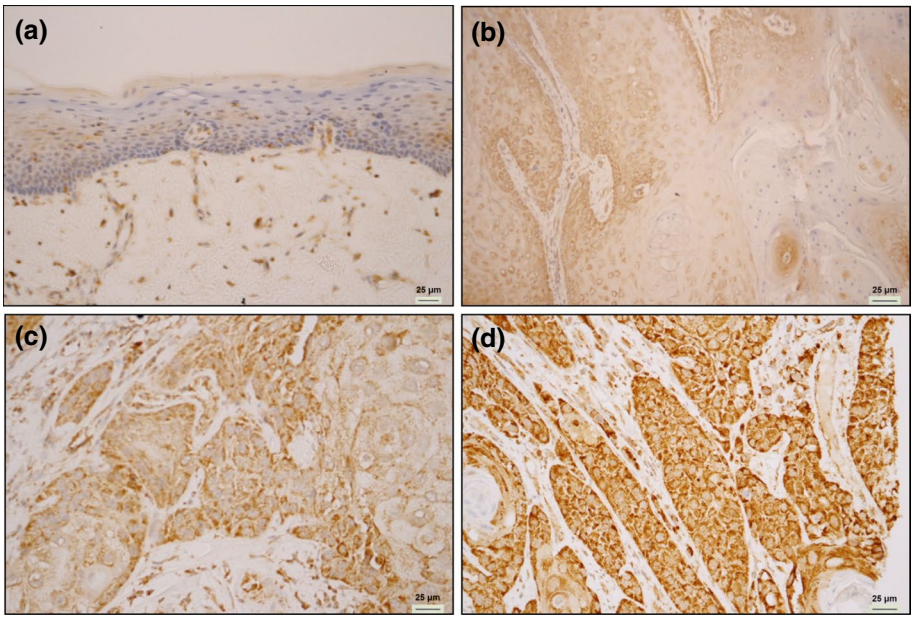


**Fig. 1** Immunohistochemical analyses of ACK1 expression in HNSCC tumors. Normal epithelia and HNSCC tumors arrayed in a tissue microarray were evaluated for the expression of ACK1 (**a**–**d**). Representative images showed **a** normal epithelia with low ACK1 expression and the HNSCC tumors with **b** low or **c**, **d** high expression of cytoplasmic ACK1. Magnification, 400x. Scale bar, 25 μm

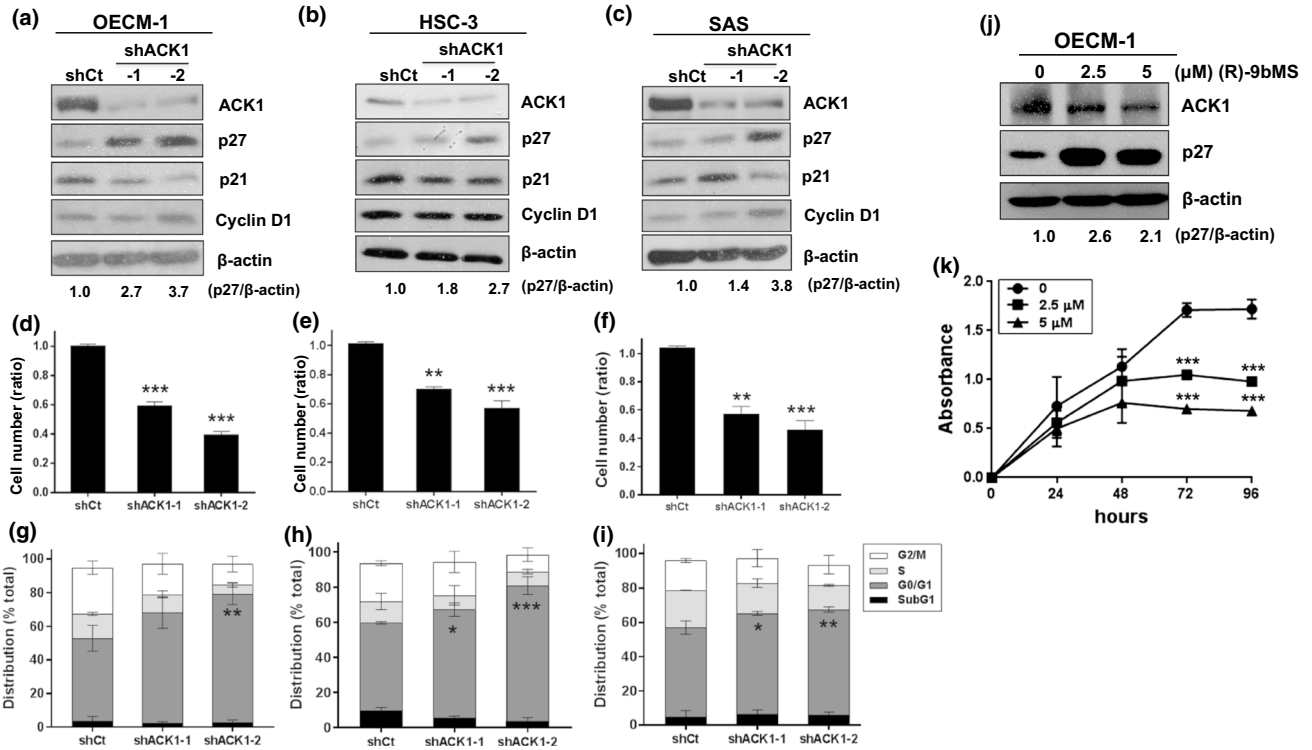


**Fig. 2** Effects of depleting ACK1 expression on the p27 expression, cell proliferation or cell cycle distribution in HNSCC cells. **a** OECM-1, **b** HSC-3, or **c** SAS cells were infected with viruses carrying control shRNA (shCt) or ACK1-targeted shRNA (shACK1-1 or shACK1-2). Infected cells were harvested and total lysates were prepared for immunoblotting. The protein levels of p27 and β-actin were quantified using ImageJ software. The relative ratio of p27 expression was normalized to the corresponding β-actin levels. Alternatively, infected **d** OECM-1, **e** HSC-3, or **f** SAS cells were seeded and cultured for 48 h. Subsequently, one set of attached cells was harvested and counted as day 0. On day 3, the number of adherent cells was counted. For flow cytometric analyses, the shCt control and ACK1-knockdown **g** OECM-1, **h** HSC-3, or **i** SAS cells were harvested and then stained with propidium iodide. The histograms showed the percentages of cells in the sub-G1, G0/G1, S, and G2/M phases of the cell cycle. **j** OECM-1 cells were treated with 2.5 or 5 μM (R)-9bMS for 24 h and the cells were harvested for western blot analyses. **k** The cell proliferation assays of (R)-9bMS-treated OECM-1 cells. Data were expressed as the mean ± SD from three independent experiments. *p<0.05, **p<0.01, ***<0.001 vs. the ratio of shCt or vehicle control cells

inconsistent with those in ACK1-knockdown OECM-1 cells (Fig. 2b, c). It suggested that ACK1 may have a less regulatory effect on p21 or cyclin D1 expression in those cells. We further performed the cell proliferation assays in ACK1-knockdown HNSCC cells. As shown in Fig. 2d, depletion of ACK1 expression in OECM-1 cells caused the inhibited proliferation, nearly 40-60% lower than that of the shCt control cells. Similarly, ACK1-knockdown HSC-3 and SAS cells showed reduced proliferation rates compared to the shCt control cells (Fig. 2e, f). Results from flow cytometric analyses further revealed that ACK1-depleted OECM-1 cells exhibited an increased population in the G0/G1 phase (66–77%), compared to the shCt control cells (50%) (Fig. 2 g). Similar results were also observed in the ACK1-knockdown HSC-3 and SAS cells (Fig. 2 h, i). We next examined whether ACK1 activity is critical for modulating p27 expression and HNSCC cell proliferation by treating cells with an ACK1 inhibitor (R)-9bMS (Wu et al. 2017). As shown in Fig. 2 J, treatment of 2.5 μM and 5 μM (R)-9bMS in OECM-1 cells caused reduced ACK1 phosphorylation but led to the increased expression of p27. Furthermore, treating cells with (R)-9bMS for 72 or 96 h significantly inhibited cell proliferation by approximately 40-60%, compared to the untreated control cells (Fig. 2 K). These data collectively suggested the functional role of ACK1 in regulating p27 expression and the proliferation in HNSCC cells.

## Rescued ACK1 expression in the ACK1-knockdown cells suppressed p27 expression and restored cell proliferation

We further examined whether the rescue of ACK1 expression in ACK1-knockdown HNSCC could suppress p27 expression and restore cell proliferation. We stably expressed a mouse ACK1 in ACK1-knockdown HNSCC cells to establish the ACK1-rescued cells. As shown in Fig. 3a (upper panel), compared with the shCt control cells, the ACK1-knockdown OECM-1 cells exhibited decreased ACK1 expression, correlating with elevated p27 expression and reduced cell proliferation (lower panel). By expressing a mouse ACK1, the elevated p27 level was greatly suppressed and reduced cell proliferation was fully restored. Similar results were also observed in the ACK1-rescued HSC-3 and SAS cells. The rescue of ACK1 expression in ACK1-knockdown cells resulted in the depletion of p27 expression and the complete recovery of cell proliferation (Fig. 3b, c). These data further supported the notion that ACK1 was critical for modulating p27 expression and the proliferation of HNSCC cells.

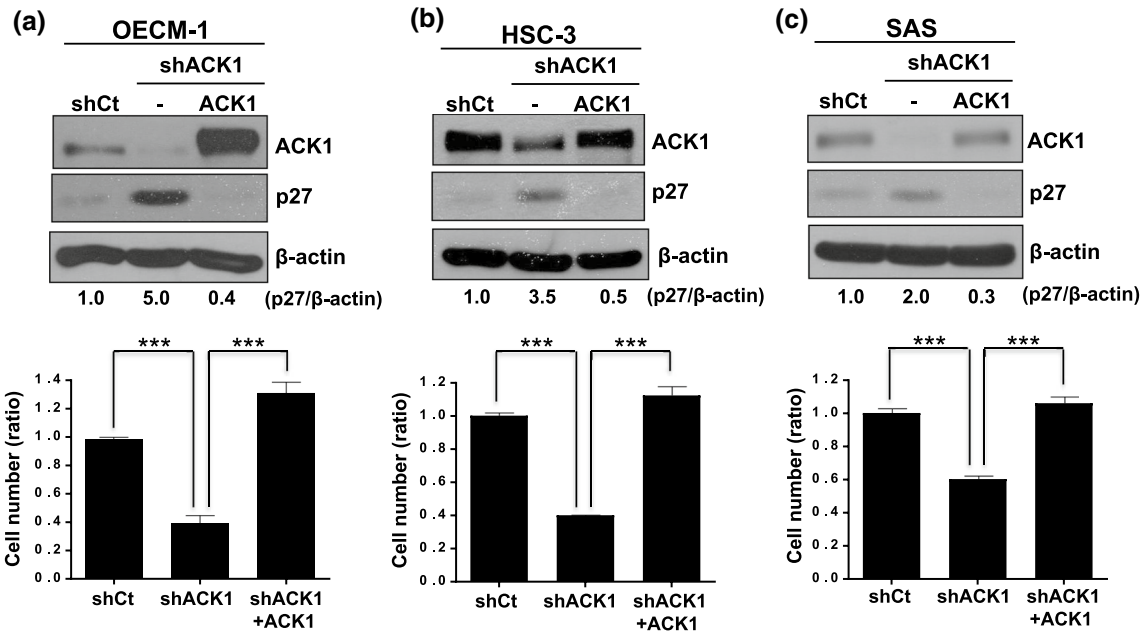


**Fig. 3** Effects of rescued ACK1 on the expression of p27 and the proliferation of ACK1-knockdown HNSCC cells.**a** OECM-1, **b** HSC-3, or **c** SAS cells infected with viruses carrying the control (shCt) or ACK1-targeted shRNA (shACK1). After 2 days of puromycin selection, the ACK1-knockdown cells were infected with the lentiviruses carrying a mouse ACK1 (shACK1+ACK1). After 4 days of incubation, cells were harvested for western blot analyses (upper panel) or seeded for cell proliferation assays (lower panel). Data were expressed as the mean ± SD from three independent experiments. ***<0.001 vs. the ratio of shCt control or shACK1 cells

## ACK1 promoted ubiquitination and proteasomal degradation of p27

To determine whether ACK1-regulated p27 downregulation is mediated by a proteasome-dependent mechanism, we treated the ACK1-rescued HNSCC cells with the proteasome inhibitor MG132 and analyzed the levels of p27. As shown in Fig. 4a, the treatment of 0.01 μM MG132 in ACK1-rescued OECM-1 cells led to the restoration of p27 levels and 0.05 μM MG132 caused a further increase in the p27 level, approximately three folds higher than that of the untreated ACK1-rescued cells. Similar results were also found in ACK1-rescued HSC-3 or SAS cells, in which treatment of MG132 restored the expression of p27 in ACK1-rescued cells (Fig. 4b, c). For further examining the role of ACK1 in enhancing the ubiquitination of p27, we expressed the Flag-tagged p27 wild-type (WT) protein in ACK1-knockdown and ACK1-rescued SAS cells. As shown

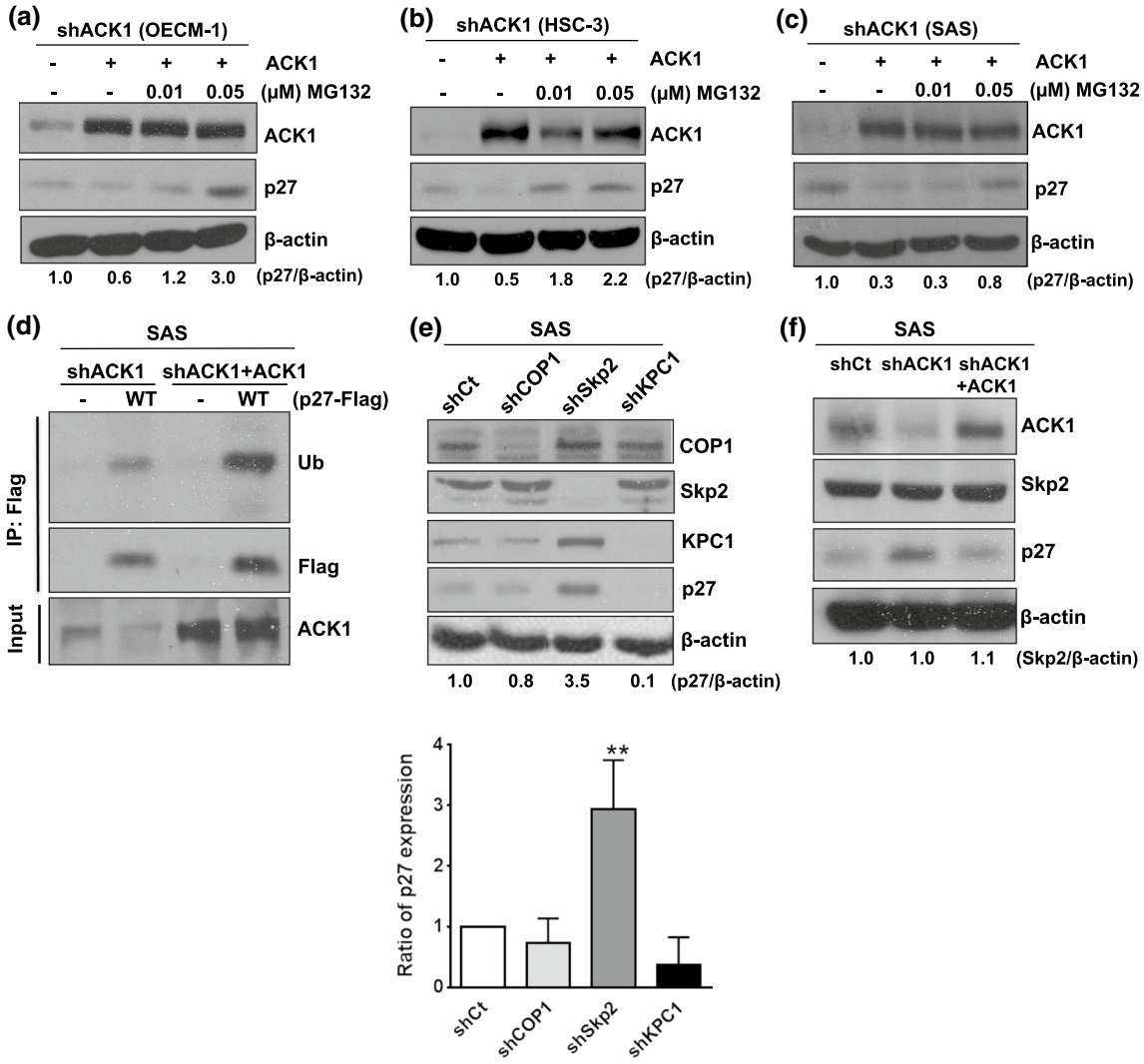


**Fig. 4** ACK1 promoted the ubiquitin-mediated proteasomal degradation of p27.The ACK1-knockdown **a** OECM-1, **b** HSC-3, or **c** SAS cells were stably expressing ACK1 and treated with 0.01 or 0.05 μM MG132 for 48 h. These cells were then harvested and total lysates were prepared for western blot analyses. **d** ACK1-knockdown (shACK1) or ACK1-rescued (shACK1+ACK1) SAS cells were transfected with the control vector or the Flag-tagged wild-type (WT) of p27 plasmids. After 48 h of culture, transfected cells were harvested and total lysates were immunoprecipitated with anti-Flag Abs. The supernatants of immunoprecipitated complexes were then immunoblotted with anti-ubiquitin (Ub) or anti-Flag Abs. Input indicates about 10% of protein amounts used for co-immunoprecipitation (co-IP). **e** SAS cells were infected with viruses carrying control shRNA (shCt) or COP1-, Skp2-, or KPC1-targeted shRNA (i.e., shCOP1, shSkp2, or shKPC1). Total lysates were prepared for western blot analyses (upper panel) and the quantification result of p27 expression was shown (lower panel). Data were expressed as the mean ± SD from three independent experiments. **<0.01 vs. the ratio of shCt control cells. **f** The shCt control, ACK1-knockdown, and ACK1-rescued SAS cells were harvested and the total lysates were prepared for western blot analyses

in Fig. 4d, the ubiquitination of exogenous p27 was markedly increased in the ACK1-rescued cells compared with the ACK1-knockdown cells. The data collectively suggested that ACK1 promoted the ubiquitin-mediated proteasomal degradation of p27 in HNSCC cells.

To examine which E3 ubiquitin ligase was involved in ACK1-promoted p27 degradation in HNSCC cells, we knocked down the expression of COP1, Skp2, or KPC1, three p27-associated E3 ubiquitin ligases (Sheaff et al. 1997; Kamura et al. 2004; Choi et al. 2015), in SAS cells. As shown in Fig. 4e, knockdown of COP1 or KPC1 in SAS cells caused decreases in itself and p27 levels. However, knockdown of Skp2 expression led to a significant increase in p27 expression. We further examined whether ACK1 enhances Skp2 expression and thus leads to p27 degradation. Compared to the shCt control cells, the levels of Skp2 were not altered in ACK1-knockdown or ACK1-rescued SAS cells, although the levels of p27 were affected (Fig. 4f). These results suggested that Skp2 played a central role in regulating p27 degradation in HNSCC cells, while the expression of Skp2 was not affected by ACK1.

## Phosphorylation of p27 at tyrosine 88 was essential for the interaction of p27 with ACK1 or Skp2

Since the tyrosine phosphorylation of p27 is critical for itself degradation, we next examined whether ACK1 could phosphorylate p27 at its tyrosine residues, including Y74, Y88, and Y89. We first co-expressed ACK1 with the wild-type or mutants of Flag-tagged p27 in 293T cells. As shown in Fig. 5a, the exogenously expressed p27(WT) in ACK1-overexpressed cells exhibited a higher tyrosyl-phosphorylation level than in the vector control cells. Furthermore, the expression of p27(Y88F) in ACK1-overexpressed cells showed a relatively low level of phosphorylation compared to the cells with the p27(WT), p27(Y74F), or p27(Y89F) expression. Importantly, the p27(WT), p27(Y74F), or p27(Y89F) showed differential levels of interaction with ACK1 and Skp2. However, the p27(Y88F) mutant was barely bound with ACK1 or Skp2, showing a basal interaction as the vector control cells. Similar results were also observed in the ACK1-knockdown and ACK1-rescued SAS cells (Fig. 5b). In the ACK1-knockdown SAS cells, the exogenously expressed p27(WT) did exhibit tyrosine phosphorylation. The expression of ACK1 in the ACK1-knockdown cells further enhanced the phosphorylation of p27(WT). Compared to the p27(WT), p27(Y74F), or p27(Y89F), the expression of p27(Y88F) in ACK1-rescued cells showed a lower level of p27 phosphorylation. Again, the p27(Y88F) mutant failed to have enhanced interaction with ACK1 or Skp2 (Fig. 5b). The data collectively suggested that ACK1 could interact with p27 and phosphorylate it predominantly at tyrosine 88, which is essential for the binding of p27 with ACK1 and Skp2.

## AKT was involved in ACK1-promoted p27 degradation

Studies have shown that ACK1 could activate AKT and AKT promoted p27 degradation. To examine whether AKT is involved in the ACK1-promoted p27 degradation, we knocked down AKT expression in ACK1-rescued

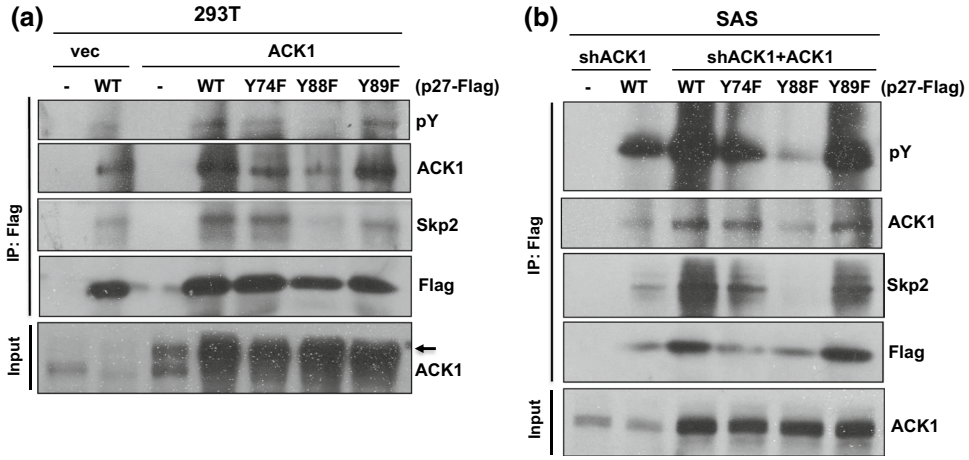


**Fig. 5** ACK1 phosphorylated and interacted with p27. **a** 293T cells were co-transfected with the control vector (vec) or ACK1 plasmids plus the Flag-tagged wild-type (WT), Y74F, Y88F, or Y89F mutant of p27 plasmids. **b** ACK1-knockdown (shACK1) or ACK1-rescued (shACK1+ACK1) SAS cells were transfected with the control vector, or the WT, Y74F, Y88F, or Y89F mutant of p27 plasmids. After 48 h of culture, transfected cells were harvested and total lysates were immunoprecipitated with anti-Flag Abs. The supernatants of immunoprecipitated complexes were then immunoblotted with anti-ACK1, anti-phosphotyrosine, or anti-Skp2 Abs. Input indicates about 10% of protein amounts used for co-IP

cells and analyzed the expression level of p27. As shown in Fig. 6a, compared to the ACK1-knockdown OECM-1 cells, ACK1-rescued cells exhibited a reduced p27 expression. However, knockdown of AKT expression in ACK1-rescued cells restored p27 expression. Similarly, knockdown of AKT expression in ACK1-rescued SAS cells caused the elevated expression of p27, compared to the control ACK1-rescued cells (Fig. 6b). These results indicated that AKT was involved in ACK1-promotes p27 degradation in HNSCC cells.

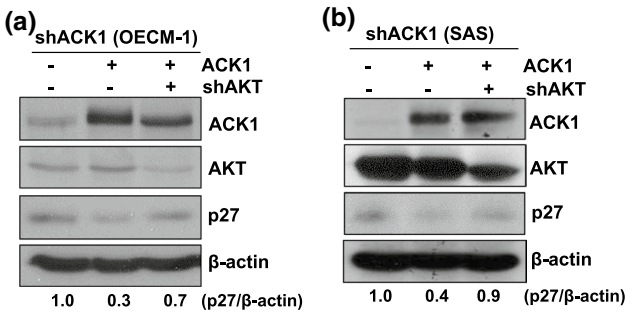


**Fig. 6** Effects of knocking down AKT expression on ACK1-promoted p27 degradation in HNSCC cells. The ACK1-knockdown **a** OECM-1 or **b** SAS cells stably expressing ACK1 were infected with viruses carrying AKT-targeted shRNA (shAKT). Cells were then harvested and total lysates were prepared for western blot analyses. The protein levels of p27 and β-actin were quantified using ImageJ software. The relative ratios of p27 expression were normalized to β-actin expression levels

## Discussion

ACK1 is an oncogenic kinase aberrantly expressed or activated in different cancers correlated with tumor progression. However, the oncogenic role of ACK1 in HNSCC cells and its underlying molecular mechanism remains unclear. In this study, we showed that ACK1 was highly expressed in HNSCC tumors and functionally regulated cell proliferation by promoting p27 phosphorylation and degradation. Our main finding was that ACK1 interacted with p27 and phosphorylated it at tyrosine 88, which was critical for recruiting Skp2 to bind with p27 and caused its degradation. The proposed model is shown in Fig. 7. To our knowledge, this is the first report showing the oncogenic role of ACK1 in HNSCC cells and its growth-promoting mechanism. Thus, ACK1 could potentially serve as therapeutic targets for HNSCC.

Accumulated evidence showed the deregulated ACK1 expression in different malignant tumors. Further studies demonstrated that gene amplification of ACK1, instead of mutation or deletion, is the major factor causing its oncogenic activation. In gastric carcinoma, the ACK1 gene is up-regulated in 22 malignant tumors compared with genes in the normal controls (Wang et al. 2006). In hepatocellular

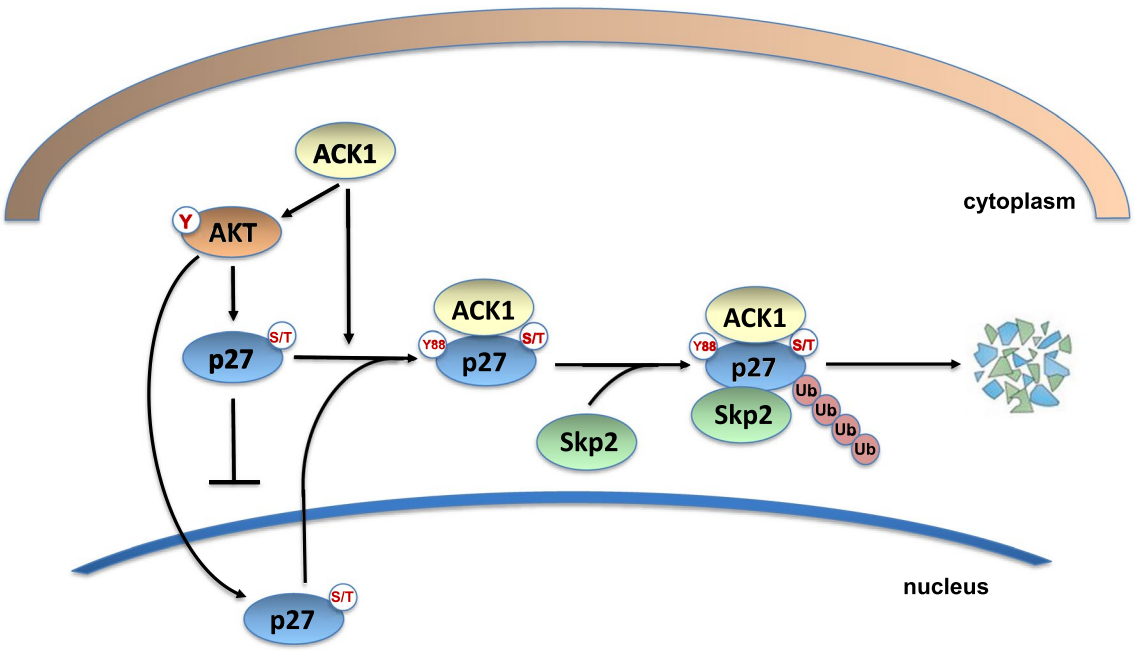


**Fig. 7** The proposed model of ACK1-regulated p27 phosphorylation and degradation in HNSCC cells. We proposed that ACK1 activates AKT activity, which causes the cytoplasmic localization of p27 by phosphorylating it at serine/threonine (S/T) residues. ACK1 could interact with p27 and phosphorylated it at tyrosine (Y) 88 residues, which led to the recruitment of Skp2 and subsequent polyubiquitination and degradation

carcinoma (HCC), both ACK1 mRNA and protein levels are significantly over-expressed in HCC tissues (Wang et al. 2014). In non-small-cell lung cancer (NSCLC), ACK1 is highly expressed in cancerous tissues compared to adjacent noncancerous tissues. In prostate carcinoma, the total ACK1 levels remain unchanged between normal and tumor samples. However, the phosphorylation level of ACK1 at Y284 in tumors is higher than those in benign tissues, correlating with disease progression (Mahajan et al. 2010a). Importantly, high expression of ACK1 in cancer cells is critical for active cell proliferation. For example, depletion of ACK1 expression or activity in triple-negative breast cancer (TNBC) cells suppresses their proliferation and tumor formation in xenograft mouse models (Wu et al. 2017). In addition, knockdown of ACK1 in HCC cells results in reduced proliferation and enhanced cell apoptosis (Xie et al. 2015). Silencing ACK1 expression in gastric cancer cells also inhibits cell proliferation and colony formation, induces G2/M arrest and cellular apoptosis, and suppresses tumor growth (Xu et al. 2017). In this study, our data first reported that ACK1 was highly expressed in HNSCC tumors and demonstrated the role of ACK1 in regulating HNSCC cell proliferation.

Studies have reported that Src family kinases, including Src, Yes, Lyn, and Brk, can phosphorylate p27 at Y74, Y88, or Y89. In addition, Abl and Jak2 phosphorylate p27 at Y88 or Y89 (Jakel et al. 2012; Patel et al. 2015). In this study, we provided evidence showing that ACK1 could be the 7th tyrosine kinase known by far to involve the phosphorylation and degradation of p27. Our data further demonstrated that ACK1 could interact with p27, and Y88 phosphorylation was critical for p27 to bind with ACK1 and recruit Skp2. Although it is unknown how ACK1 could interact with p27, a study showed that p27 structurally exhibits three proline-rich sequences (K1, K2, and K3) containing the PXXP core motif. Moreover, Abl, Yes, or Brk interacts with the K3 region of p27 via their SH3 domain. Interestingly, Brk also binds weakly to the K1 region, which is essential for Brk's phosphorylation of p27 at Y88 (Patel et al. 2015). Thus, ACK1 may be via its SH3 domain to interact with the proline-rich sequences of p27 in HNSCC cells.

Based on the model proposed by Galea et al. (Galea et al. 2008), tyrosine kinases phosphorylate the residue Y88 of p27 bound to cyclin A/Cdk2 in the nucleus. It leads to the activation of Cdk2 and phosphorylation of p27 at T187, resulting in the Skp2-mediated p27 ubiquitination and proteasomal degradation. Thus, It is possible that nuclear ACK1 interacted with the Cdk2-bound p27 and phosphorylated it, causing the subsequently T187 phosphorylation, Skp2 recruitment, and p27 degradation. However, our IHC data clearly showed that ACK1 was predominantly expressed in the cytoplasm of HNSCC cells. In other words, ACK1 may drive p27 phosphorylation and Skp2 recruitment in the cytoplasm of HNSCC cells. This notion was supported by the observations that the Y88-phosphorylated p27 is highly abundant in the cytoplasm of cancer cells (Jakel et al. 2011). In addition, the cytoplasmic expression of Skp2 is found in HNSCC cells (Yamada et al. 2016). Furthermore, our data clearly showed that AKT was involved in the ACK1-promoted p27 degradation. According to the literature, ACK1 can interact with AKT and phosphorylate it at Y176, causing AKT activation in a PI3K-independent fashion (Mahajan et al. 2010b; Mahajan and Mahajan 2010). In addition, AKT can phosphorylate p27 at S10, T157, or T198, which caused the nuclear export or cytoplasmic retention of p27 (Hnit et al. 2015). AKT also functions to promote the cytoplasmic localization of Skp2 by phosphorylating it (Gao et al. 2009). Thus, ACK1 might activate AKT to promote the cytoplasmic localization p27 and Skp2. It allowed ACK1 to bind and phosphorylate p27, thus causing Skp2 recruitment and p27 degradation.

In summary, we demonstrated that ACK1 was highly expressed in HNSCC tumors. We further showed that ACK1-enhanced proliferation in HNSCC cells was associated with the proteasomal degradation of p27. Importantly, ACK1 could bind to p27 and phosphorylate it at Y88, leading to Skp2 recruitment. Thus, our data supported that ACK1 could serve as therapeutic targets for the treatment of HNSCC.

**Acknowledgements** We would like to thank Dr. Kuo-Wei Chang at National Yang-Ming University (Taipei, Taiwan) for kindly providing experimental materials. This work was supported by a grant from the Taiwan National Science Council (NSC MOST 107-2314-B-259-001).

## Declarations

**Conflict of interest** The authors declare no conflict of interest.

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