

IL-17A promotes the proliferation of human nasopharyngeal carcinoma cells through p300-mediated Akt1 acetylation

KEMIN CAI^{1*}, BING WANG^{2*}, HONGMEI DOU^{1*}, RONGLAN LUAN^{1*}, XUELI BAO^{1*} and JIUSHENG CHU¹

¹Department of Otorhinolaryngology Head and Neck Surgery, Taizhou People's Hospital, Taizhou, Jiangsu 225300; ²Department of Neurosurgery, Suzhou Kowloon Hospital Affiliated with Shanghai Jiao Tong University School of Medicine, Suzhou, Jiangsu 215021, P.R. China

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Abstract. Interleukin (IL)-17A is a T helper (Th)17 cell-secreted cytokine that is able to induce various inflammatory responses. There is emerging evidence that IL-17A is generated in the cancer microenvironment of human nasopharyngeal carcinoma (NPC). However, the role of IL-17A in NPC remains unclear. Thus, the present study aimed to examine the direct influence of IL-17A stimulation on the proliferation of human NPC cells and identify the underlying molecular mechanisms. Furthermore, E1A binding protein p300 (p300)-mediated AKT serine/threonine kinase 1 (Akt1) acetylation and its role in regulating the proliferation of NPC cells was investigated. The results of the current study demonstrated that IL-17A stimulation *in vitro* increased the proliferation of human NPC cells. Furthermore, Akt1 acetylation was identified to be enhanced in human NPC cells induced by IL-17A. Additionally, p300 induction was demonstrated to be required for Akt1 acetylation in human NPC cells following exposure to IL-17A. Functionally, p300-mediated Akt1 acetylation contributed to the proliferation of human NPC cells stimulated by IL-17A. In conclusion, the results of the present demonstrate a novel activity of IL-17A that promotes human NPC cell proliferation via p300-mediated Akt1 acetylation. This may provide a potential strategy for the treatment of patients with NPC through the inhibition of IL-17A or its receptors.

Introduction

Nasopharyngeal carcinoma (NPC) is a common tumor that occurs in the head and neck. NPC is caused by a

combination of factors including viral, environmental and heredity reasons (1-5). However, its pathogenesis remains unclear. Previous studies have demonstrated that T helper (Th)17 cells and Th17 cell-secreted interleukin (IL)-17A in the tumor microenvironment leads to a significant increase in tumor progression of various types of cancer, including lung, intestinal and brain cancer (6-8). Furthermore, it has been reported that exogenous IL-17A may significantly promote cell migration and invasion in NPC cells (9); however, the effect of IL-17A on NPC cells and its underlying mechanism remain unclear.

It is well-known that the activation of signaling pathways is involved in various types of carcinoma (10-13). Among these, AKT serine/threonine kinase 1 (Akt1) activation has been revealed to serve an essential role in numerous types of carcinoma, including NPC (14-21). Thus, targeting aberrant Akt1 signaling may provide an effective and novel strategy for the treatment of patients with NPC. Despite the fact that Akt1 activation is common in NPC, the mechanism of this activation has not been elucidated.

Increasing evidence suggests that modification of protein acetylation is involved in the regulation of various cellular biological events, including cell proliferation, apoptosis, autophagy and inflammatory responses (22-26). It has been reported that Akt1 acetylation promotes Akt1 phosphorylation and enhances the proliferation of human glioblastoma cells (27); however, the role of Akt1 acetylation in regulating Akt1 activation in NPC remains unclear.

In the current study, the role of IL-17A in the proliferation of human NPC cells was evaluated *in vitro*. E1A binding protein p300 (p300)-mediated Akt1 acetylation and its role in the regulation of NPC cell proliferation were also investigated.

Materials and methods

Reagents. Recombinant human IL-17A was obtained from R&D Systems, Inc. (Minneapolis, MN, USA). Monoclonal antibodies against human CREB-binding protein (CBP; cat. no. sc-7300; dilution, 1:400), p300 (cat. no. sc-48343; dilution, 1:500) and p300/CBP-associated factor (PCAF; cat. no. sc-13124; dilution, 1:500) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Monoclonal antibodies against human β -actin (cat. no. 4970; dilution, 1:1,000), total

Correspondence to: Dr Jiusheng Chu, Department of Otorhinolaryngology Head and Neck Surgery, Taizhou People's Hospital, 210 Yingchun Road, Taizhou, Jiangsu 225300, P.R. China
E-mail: chujiushengsz@sina.com

*Contributed equally

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Akt1 (t-Akt1; cat. no. 2938; dilution, 1:1,000), phosphorylated (p)-Akt-Thr³⁰⁸ (cat. no. 13038; dilution, 1:1,000), p-Akt-Ser⁴⁷³ (cat. no. 4060; dilution, 1:2,000) and acetylated-lysine (cat. no. 9681; dilution, 1:1,000) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase-conjugated anti-rabbit (cat. no. 7074; dilution, 1:3,000), anti-mouse (cat. no. 7076; dilution, 1:3,000) and anti-rabbit (Conformation Specific; cat. no. 5127; dilution, 1:2,000) immunoglobulin G (IgG) secondary antibodies, blocking skim-milk and the enhanced chemiluminescence detection system were purchased from Cell Signaling Technology, Inc. Polyvinylidene difluoride membranes were obtained from EMD Millipore (Billerica, MA, USA). Anti-IL-17AR neutralizing antibody (cat. no. ab92608) and iso control IgG (cat. no. ab81032) were purchased from Abcam (Cambridge, UK). The plasmids of pGCsi-U6/neo/green fluorescent protein (GFP) were obtained from Shanghai Genkan Biotechnology Co., Ltd. (Shanghai, China). The pcDNA3.1 vector and Lipofectamine[®] 2000 were obtained from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). A QIAprep spin miniprep kit was obtained from Qiagen GmbH (Hilden, Germany). The Cell Counting Kit-8 (CCK-8) assay was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan).

Cell culture. The human NPC cell line C666-1 was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) fetal bovine serum from Gibco (Thermo Fisher Scientific, Inc.), 50 U/ml penicillin and 100 µg/ml streptomycin (both Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in 5% CO₂.

Cell treatment and study design. Various doses of purified human IL-17A protein (0, 0.001, 0.01, 0.1, 1, 10 and 100 ng/ml) were added into the culture medium of C666-1 NPC cells. Following 48 h incubation at 37°C, cellular proliferation was detected using the CCK-8 assay. Next, the cells were incubated with anti-IL-17AR neutralizing antibody (0.01, 0.1 and 1 µg/ml) or iso control IgG (0.01, 0.1 and 1 µg/ml) at 37°C for 30 min, and subsequently incubated with IL-17A (10 ng/ml) or PBS solvent control at 37°C for 48 h. Subsequently, cellular proliferation was detected. The levels of Akt1 phosphorylation at Thr308 and Ser473 sites, as well as Akt1 acetylation were detected in C666-1 cells stimulated with IL-17A (10 ng/ml) or PBS solvent control at 37°C for 30 min. Furthermore, the expression of CBP, p300 and PCAF, as well as the interaction of Akt1 with CBP, p300 and PCAF were detected in C666-1 cells stimulated with IL-17A (10 ng/ml) or PBS solvent control at 37°C for 30 min. C666-1 cells were transfected with p300 short hairpin (sh)RNA or control shRNA and cultured at 37°C for 48 h, followed by IL-17A stimulation (10 ng/ml) at 37°C for 30 min or 48 h. Akt1 acetylation and phosphorylation in C666-1 cells were determined at 30 min following IL-17A stimulation, and cellular proliferation was determined at 48 h following IL-17A stimulation. Finally, Ly294002 (10 µM/ml) or DMSO solvent control were added into the culture medium of C666-1 NPC cells at 30 min prior to IL-17A stimulation (10 ng/ml) and the cellular proliferation was detected at 48 h following IL-17A stimulation.

Generation of overexpression plasmids. The plasmid of pcDNA3.1/Akt1-His was provided by Dr. Lei Shi at The First People's Hospital of Kunshan affiliated to Jiangsu University (Zhenjiang, China).

Construction of (sh)RNA expression plasmids. To silence the p300 gene in human NPC cells, various shRNA sequences were designed against human p300 mRNA (NM_001429.3) by Shanghai Genkan Biotechnology Co., Ltd. The different DNA segments of p300 shRNA were constructed into the plasmids of pGCsi-U6/neo/GFP respectively by Shanghai Genkan Biotechnology Co., Ltd. and the most effective shRNA expression plasmids (5'-GGACAGGTATCAAATCCTC-3') were selected for further functional experiments.

Cellular transfection. NPC cells were transfected with pcDNA3.1/Akt1-His or pGCsi-U6/neo/GFP using Lipofectamine 2000 according to a previously described method (28). Cells were seeded into 6-well plates at a density of 1x10⁵ cells/well. Following 24 h at 37°C, 4 µg of plasmid was transfected into each well.

Co-immunoprecipitation experiment. For certain studies, co-immunoprecipitation experiments were performed using an antibody against t-Akt1 according to a previously described method (28).

Western blot analysis. Western blot analysis was performed to detect β-actin, CBP, p300, PCAF, t-Akt1, p-Akt-Thr³⁰⁸, p-Akt-Ser⁴⁷³ and acetylated-lysine, according to a previously described method (28).

CCK-8 assay. Following each treatment, NPC cells were incubated with CCK-8 at 37°C for 4 h. The formazan product was measured with a microplate reader at a wavelength of 450 nm and the absorbance was directly proportional to the cell number (29).

Statistical analysis. All statistical analysis was performed using SPSS software (version 11.5; SPSS, Inc., Chicago, IL, USA). All data are presented as the mean ± standard deviation. The statistical significance of groups was evaluated using one-way analysis of variance with simultaneous multiple comparisons between groups using Bonferroni's correction. P<0.05 was considered to indicate a statistically significant difference.

Results

IL-17A promotes the proliferation of human NPC cells. Various doses of purified human IL-17A protein (0, 0.001, 0.01, 0.1, 1, 10 and 100 ng/ml) were added into the culture medium to induce proliferation of C666-1 NPC cells at 37°C for 48 hr. The proliferation assay demonstrated that IL-17A enhanced the proliferation of C666-1 cells in a dose-dependent manner (Fig. 1A). Compared with the untreated control group, the proliferative activity was significantly increased in the C666-1 cells treated with 0.1 ng/ml IL-17. Furthermore, the cells were incubated with anti-IL-17AR neutralizing antibody or IgG iso control at 37°C for 30 min and subsequently incubated with

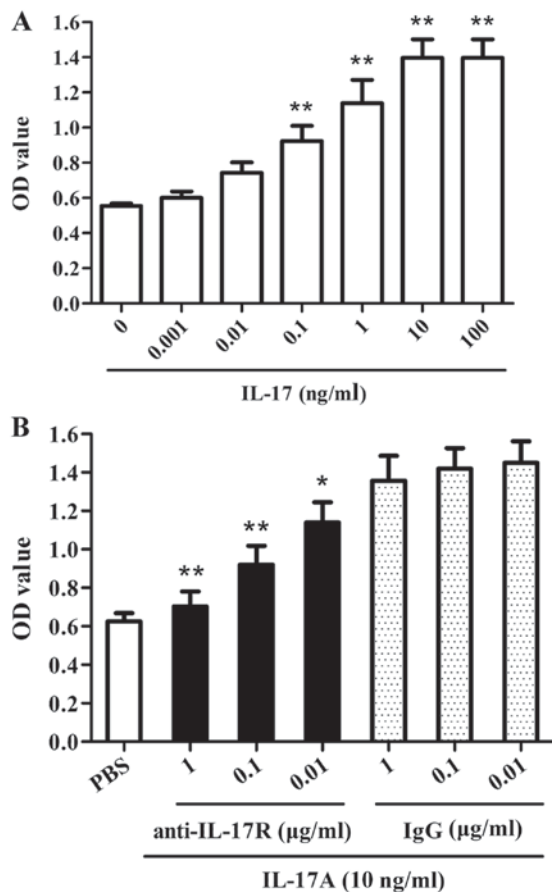


Figure 1. Effect of IL-17A on the proliferation of nasopharyngeal carcinoma C666-1 cells. (A) C666-1 cells were treated with various doses (0.001-100 ng/ml) of human IL-17A to induce cellular proliferation for 48 h. CCK-8 analysis revealed that IL-17A stimulation enhanced the proliferation of C666-1 cells in a dose-dependent manner *in vitro*. ** $P < 0.01$ vs. untreated control group. (B) C666-1 cells were incubated with various doses of anti-IL-17AR neutralizing antibody or isotype control IgG for 30 min, and subsequently cellular proliferation was induced by 10 ng/ml IL-17A for 48 h. CCK-8 analysis demonstrated that blockade of the IL-17A receptor suppressed the IL-17A-triggered proliferation of C666-1 cells in a dose-dependent manner. * $P < 0.05$, ** $P < 0.01$ vs. IgG+IL-17A group. The data are representative of three independent experiments. All results are represented as the mean \pm standard deviation. IL-17A, interleukin-17A; CCK-8, Cell Counting Kit-8; OD, optical density; IgG, immunoglobulin G.

IL-17A or PBS control at 37°C for 48 h. The proliferation assay revealed that blockade of the IL-17A receptor significantly reduced IL-17A-triggered C666-1 cell proliferation compared with the isotype IgG control group (Fig. 1B). These findings indicate that IL-17A is able to promote the proliferation of human NPC cells.

Akt1 phosphorylation and acetylation is enhanced in human NPC cells induced by IL-17A. Since Akt1 activation is considered to be involved in human NPC cell proliferation (14-17), the effect of IL-17A stimulation on Akt1 activation was further investigated in NPC cells. It was demonstrated that the levels of Akt1 phosphorylation at Thr³⁰⁸ and Ser⁴⁷³ sites were markedly enhanced in C666-1 cells stimulated by IL-17A for 30 min *in vitro* compared with the untreated control group (Fig. 2A). It has been reported that the modification of protein acetylation is an important post-transcriptional regulation that may promote Akt1 phosphorylation (27). Therefore, the

effect of IL-17A stimulation on Akt1 acetylation was studied in C666-1 cells subsequently. The result revealed that the level of Akt1 acetylation was markedly increased in human NPC cells following stimulation with IL-17A for 30 min *in vitro* compared with the isotype IgG treated control group (Fig. 2B).

Interaction of p300 with Akt1 is increased in human NPC cells treated with IL-17A. In order to investigate the potential mechanism underlying Akt1 acetylation, the expression of CBP, p300 and PCAF (three important acetyl transferases) (30-32) and the interaction of Akt1 with CBP, p300 and PCAF at the protein level were detected in C666-1 cells stimulated by IL-17A for 30 min. It was demonstrated that IL-17A did not markedly enhance the lysate protein expression of CBP, p300 and PCAF in C666-1 cells compared with the corresponding untreated control groups (Fig. 3). However, the interaction of Akt1 with p300, but not with CBP or PCAF at the protein level, was revealed to be markedly enhanced in C666-1 cells in response to IL-17A stimulation compared with the untreated and isotype IgG control groups (Fig. 3). These results indicate that IL-17A stimulation enhances the interaction of p300 with Akt1 at the protein level in human NPC cells, suggesting a potential role of p300 in acetylating Akt1.

p300 is required for Akt1 acetylation in NPC cells by exposure to IL-17A. In order to further determine the role of p300 in Akt1 acetylation and phosphorylation, C666-1 cells were treated with shRNA targeted against p300 for 48 h, followed by IL-17A stimulation for 30 min. The co-immunoprecipitation assay demonstrated that the shRNA knockdown of p300 significantly suppressed Akt1 acetylation and phosphorylation in C666-1 cells exposed to IL-17A, compared with the corresponding shRNA control groups (Fig. 4), suggesting that the acetyltransferase activity of p300 is required for Akt1 acetylation and phosphorylation in NPC cells.

p300-mediated Akt1 acetylation contributes to the proliferation of NPC cells stimulated by IL-17A. Since p300 was revealed to be required for Akt1 acetylation in NPC cells by exposure to IL-17A (Fig. 4), the role of p300-mediated Akt1 acetylation in proliferation of NPC cells stimulated by IL-17A was further determined. The results demonstrated that the shRNA knockdown of p300 (Fig. 5A) or the blockade of Akt1 via Ly294002 treatment (Fig. 5B) could cause significant inhibition with regards to cellular proliferation in C666-1 cells treated with IL-17A. Taken together, the data indicate that p300-mediated Akt1 acetylation contribute to the proliferation of NPC cells stimulated by IL-17A.

Discussion

NPC is one of the most common types of malignancy in Southern China and Southeast Asia (1,2). However, the molecular pathogenesis of NPC remains unclear. Recently, inflammatory response has been considered to serve an important role in the process of cancer development (33-36). Furthermore, the generation of cytokines, particularly IL-17A, has been demonstrated to serve an essential role in regulating inflammatory responses *in vivo* (37,38) and contribute to cancer progression (39,40). In the current study, the effect of IL-17A

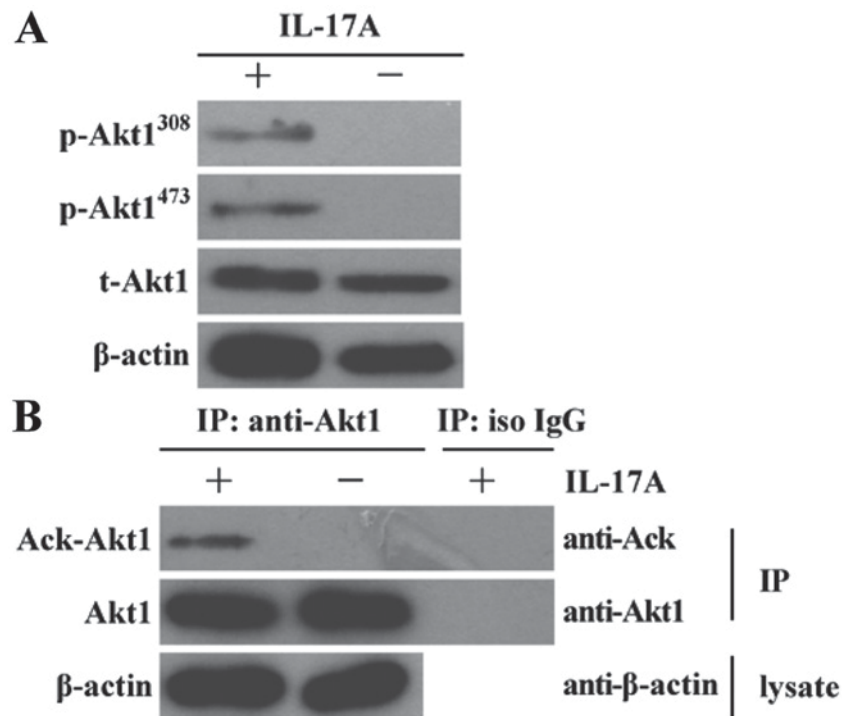


Figure 2. Effect of IL-17A on Akt1 phosphorylation and acetylation in nasopharyngeal carcinoma C666-1 cells. (A) C666-1 cells were stimulated with 10 ng/ml IL-17A for 30 min, and subsequently Akt1 phosphorylation was detected by western blot analysis. The results demonstrated that the levels of Akt1 phosphorylation at the Thr³⁰⁸ and Ser⁴⁷³ sites were markedly increased in C666-1 cells induced by IL-17A. (B) C666-1 cells were incubated with 10 ng/ml IL-17A for 30 min, and subsequently anti-Akt1 antibody and isotype control IgG were used respectively to perform immunoprecipitation following cell lysis. Subsequently, Ack-Akt and total Akt1 in immunoprecipitation complex were detected by western blot analysis. The results revealed that the level of Akt1 acetylation was markedly enhanced in C666-1 cells following stimulation with IL-17A compared with untreated control and iso IgG treated control groups. The data are from one experiment (n=3 in each group), and are representative of three independent experiments. Representative western blot photographs were produced. IL-17A, interleukin-17A; IgG, immunoglobulin G; Ack, acetylated; Akt1, AKT serine/threonine kinase 1; p, phosphorylated; t, total; iso, isotype; IP, immunoprecipitation.

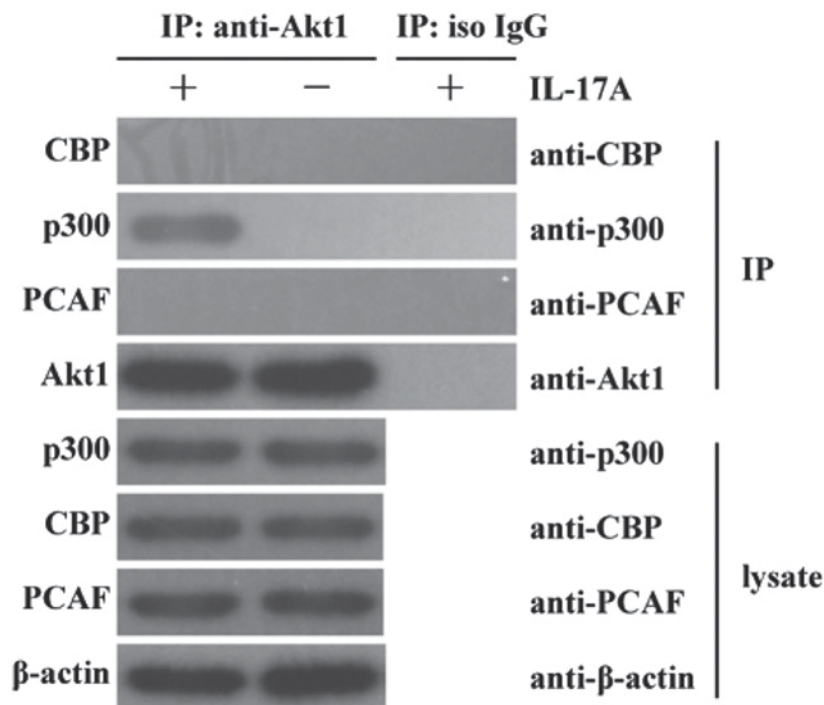


Figure 3. Association of Akt1 with p300 in IL-17A-treated nasopharyngeal carcinoma cells. C666-1 cells were stimulated with 10 ng/ml IL-17A for 30 min. Western blot analysis revealed that the expression of p300, CBP and PCAF was not induced by IL-17A stimulation. Further co-immunoprecipitation analysis demonstrated that the interaction of Akt1 with p300, but not with CBP or PCAF, was induced in C666-1 cells in response to IL-17A stimulation for 30 min. The data are from one experiment (n=3 in each group), and are representative of three independent experiments. Representative western blot photographs were produced. IL-17A, interleukin-17A; IgG, immunoglobulin G; CBP, CREB-binding protein; p300, E1A binding protein p300; PCAF, p300/CBP-associated factor; Akt1, AKT serine/threonine kinase 1; iso, isotype; IP, immunoprecipitation.

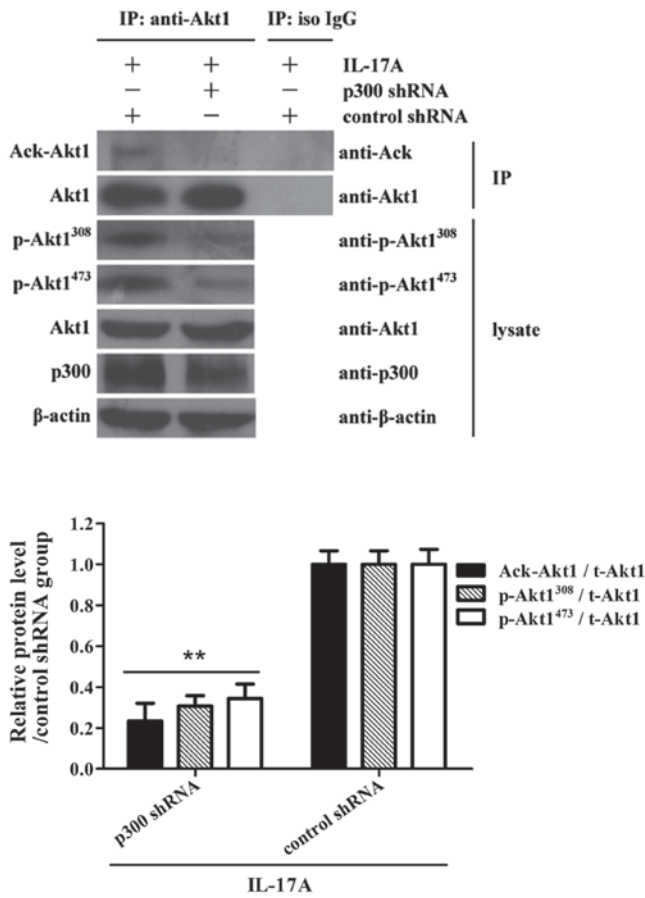


Figure 4. Role of p300 in Akt1 acetylation and phosphorylation in nasopharyngeal carcinoma cells following exposure to IL-17A. C666-1 cells were transfected with p300 shRNA or control shRNA for 48 h prior to IL-17A stimulation. Co-immunoprecipitation and western blot analysis were further used to detect Akt1 acetylation and phosphorylation at 30 min following IL-17A stimulation. The results showed that p300 shRNA suppressed Akt1 acetylation and phosphorylation in C666-1 cells following exposure to IL-17A compared to the control shRNA treatment. **P<0.01 vs. control shRNA+IL-17A group. The data are from one experiment, and are representative of three independent experiments. Results are presented as the mean ± standard deviation (n=3 in each group). Representative western blot photographs were produced. IL-17A, interleukin-17A; IgG, immunoglobulin G; Ack, acetylated; Akt1, AKT serine/threonine kinase 1; p, phosphorylated; t, total; iso, isotype; IP, immunoprecipitation; p300, E1A binding protein p300; sh, short hairpin.

in the proliferation of human NPC cells was initially evaluated. It was revealed that IL-17A *in vitro* was able to enhance the proliferation of human NPC cells. Further investigation revealed that blockade of the IL-17A receptor was able to inhibit IL-17A-triggered NPC cell proliferation. These results indicate that an overproduction of IL-17A may contribute to the proliferation of human NPC cells. Notably, Li *et al* (41) identified that tumor microenvironment macrophage inhibitory factor directs the accumulation of IL-17-producing tumor-infiltrating lymphocytes and predicts favorable survival in patients with NPC. However, the results of the present study suggest that IL-17A directly promotes the proliferation of human NPC cells. One possible explanation is that tumor microenvironment macrophages inhibit NPC growth via other mechanisms, although they may promote the accumulation of IL-17-producing tumor-infiltrating lymphocytes within NPC tumor tissue.

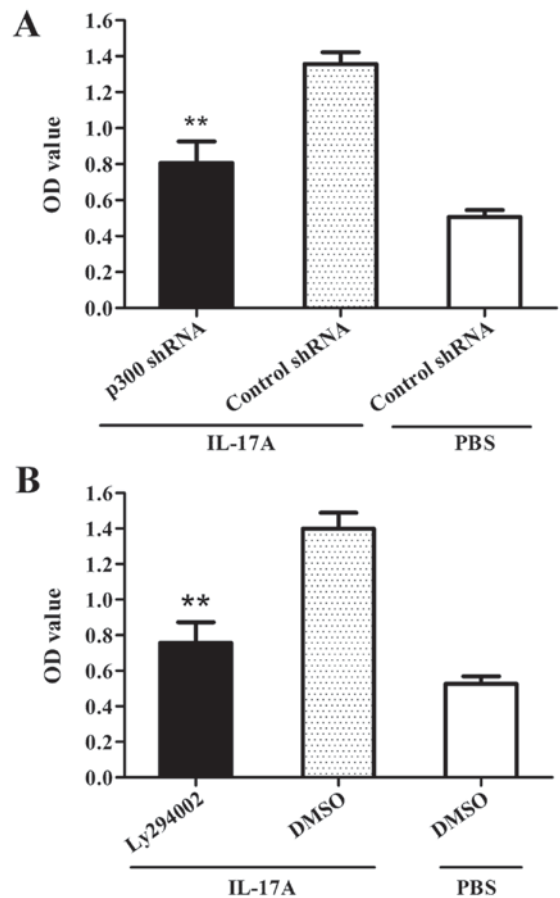


Figure 5. Role of p300-mediated Akt1 acetylation in the proliferation of nasopharyngeal carcinoma cells stimulated by IL-17A. (A) C666-1 cells were transfected with p300 shRNA or control shRNA for 48 h, followed by IL-17A stimulation for an additional 48 h, and cell proliferation was detected by CCK-8 assay. The results showed that p300 shRNA treatment was able to inhibit the proliferation of C666-1 cells induced by IL-17A compared to control shRNA treatment. **P<0.01 vs. control shRNA+IL-17A group. (B) CCK-8 analysis revealed that C666-1 cells treated with the protein inhibitor Ly294002 for 30 min exhibited reduced cellular proliferation in response to IL-17A stimulation for 48 h compared to C666-1 cells treated with DMSO. **P<0.01 vs. DMSO+IL-17A group. The data are from one experiment, and are representative of three independent experiments. Results are presented as the mean ± standard deviation (n=3 in each group). sh, short hairpin; OD, optical density; p300, E1A binding protein p300; DMSO, dimethyl sulfoxide; IL-17A, interleukin-17A; Akt1, AKT serine/threonine kinase 1.

The proliferation of cancer cells is regulated by a complex array of signaling pathways (42,43). Among these signaling pathways, the activation of Akt1 is involved in proliferative and anti-apoptotic effects in NPC (44,45). Therefore, in the present study, Akt1 phosphorylation was further measured in NPC cells stimulated with IL-17A. The data revealed that Akt1 phosphorylation was increased in NPC cells treated with IL-17A compared with the untreated control group. However, the mechanism underlying the regulation of Akt1 phosphorylation remains elusive. Given that Akt1 is able to undergo acetylation, which is an essential regulatory mechanism required for Akt phosphorylation and subsequent activation (27), Akt1 acetylation was further measured in NPC cells stimulated by IL-17A. The results of the present study demonstrated that the level of Akt1 acetylation was markedly elevated in human NPC cells treated with IL-17A compared with the isotype IgG treated control group.

Since Akt1 acetylation was markedly enhanced in human NPC cells treated with IL-17A, further experiments were designed to investigate the mechanism of Akt1 acetylation. It is well accepted that CBP, p300 and PCAF possess acetyl transferase activity and serve direct roles in the regulation of transcription factor and signaling molecule activity (30-32). Therefore, the roles of CBP, p300 and PCAF in Akt1 acetylation were investigated in C666-1 cells treated with IL-17A. The results revealed that the interaction of Akt1 with p300, but not CBP and PCAF at the protein level, was enhanced in C666-1 cells in response to IL-17A stimulation. In addition, p300 silencing suppressed Akt1 acetylation and phosphorylation, and the cellular proliferation of NPC cells in response to IL-17A. These results indicate that p300-mediated Akt1 acetylation is important for Akt1 activation and cellular proliferation in human NPC cells induced by IL-17A. Since Akt activation is regulated by a complex mechanism including phosphorylation, ubiquitination, SUMOylation and acetylation (27,46,47), further studies are required to investigate the potential interaction of these modifications in human NPC cells exposed to IL-17A.

In conclusion, the results of the current study revealed that IL-17A could stimulate the proliferation of human NPC cells *in vitro*. Furthermore, it was demonstrated that p300-mediated Akt1 acetylation was involved in the proliferation of human NPC cells in response to IL-17A. These results may provide a potential strategy for treating patients with NPC through the inhibition of IL-17A or its receptors.

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