CBP-mediated acetylation of NF-κB p65 contributes to the proliferation of human nasopharyngeal carcinoma cells

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Abstract

Introduction: Reportedly, activation of the NF-κB signaling pathway is related to the proliferation of nasopharyngeal carcinoma (NPC) cells; however, the underlying mechanism by which NF-κB is activated in NPC cells is yet to be addressed. In our present studies, CREB-binding protein (CBP)-mediated acetylation of the NF-κB p65 subunit and its effect on activation of NF-κB p65 as well as its role in the proliferation of NPC cells were explored.
Material and methods: Human NPC cell lines CNE1 and C666-1 were cultured *in vitro*. The acetylation (Lys310) and phosphorylation (Ser536) levels of NF-κB p65 in NPC cell lines were detected by Western blot. Then, the mRNA levels of CBP, p300, PCAF and GCN5 in NPC cell lines were determined by real-time PCR. Subsequently, the interaction of p65 with CBP in NPC cell lines was detected by a co-immunoprecipitation experiment. Furthermore, an NF-κB p65 mutant (at Lys310), NF-κB inhibitor and CBP shRNA were used to study the effect of CBP-mediated acetylation of NF-κB p65 on the proliferation of NPC cell lines by CCK-8 assay.
Results: NF-κB p65 was markedly acetylated at the site of Lys310 in NPC cells. Moreover, the mutation of NF-κB p65 at the acetylation site markedly reduced proliferation of human NPC cells. Further investigation revealed that CBP was up-regulated and physically associated with NF-κB p65 at the protein level in NPC cells. CBP-mediated NF-κB p65 acetylation enhanced its phosphorylation and further contributed to the proliferation of NPC cells.
Conclusions: This study indicated that CBP-mediated NF-κB p65 acetylation promotes the proliferation of NPC cells.

Key words: nasopharyngeal carcinoma, proliferation, NF-κB p65, acetylation, CREB-binding protein.

Introduction

Nasopharyngeal carcinoma (NPC) is an epithelial derived carcinoma located in the nasopharynx, with relative high prevalence in southern China and other Southeast Asian countries [1–3]. Reportedly, genetic susceptibility and environmental factors play important roles in NPC formation [4–8]; however, the pathogenesis of human NPC remains largely unclear.

It is well known that NF-κB activation plays pivotal roles in the proliferation of various types of carcinomas including NPC [9–15]. Neverthe-

less, to date, limited reports have presented the activation mechanism of canonical NF-κB in NPC cells. As a posttranslational modification, protein acetylation exhibits effects on various cellular biological events such as apoptosis and proliferation [16–21]. Multiple studies have revealed that the acetylation modification of NF-κB p65 subunit regulates its transcriptional activity [22–25]. Notably, NF-κB p65 acetylation is able to promote its phosphorylation and enhance the proliferation of some types of carcinoma cells such as gastric cancer, breast cancer and lung cancer [26–28]; however, the acetylation of NF-κB p65 and its roles in NPC are still unknown.

CREB-binding protein (CBP) is not only a transcriptional co-activator but also a common acetyltransferase. Reportedly, CBP not only acetylates histones but also acetylates non-histone proteins [29, 30], and thus plays important regulatory roles in embryonic development and cell apoptosis, proliferation and differentiation [31–34]. Although CBP expression is reportedly involved in the development of lung cancer and liver cancer [31, 35], and CBP is able to promote the acetylation of NF-κB p65 [36], the expression of CBP and its regulatory effect on NF-κB p65 acetylation in NPC cells remain largely unclear.

In our current study, the acetylation of NF-κB p65 and its role in cell proliferation were determined in human NPC cells. Subsequently, the role of CBP in NF-κB p65 acetylation as well as the effect of CBP-mediated NF-κB p65 acetylation on cellular proliferation was explored in human NPC cells.

Material and methods

Reagents

Rabbit monoclonal antibodies against human total p65, phosphorylated-p65 (p-p65) and acetyl-p65 (Lys310) as well as monoclonal mouse antibody against human β-actin were from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal antibodies against human p300, CBP and His-tag were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). For western blot analysis, horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody and anti-rabbit IgG antibody as well as chemiluminescence ECL (20X LumiGLO Reagent and 20X Peroxide) were from Cell Signaling Technology. Co-immunoprecipitation (co-IP) assay buffer and protein G-Sepharose beads were purchased from Thermo Fisher Scientific (Waltham, MA, USA). A BCA protein assay kit was from Beyotime Biotechnology (Nantong, China). TRIzol reagent was obtained from Invitrogen (Thermo Fisher Scientific). A RevertAid First Strand cDNA Synthesis Kit and TaqMan Fast Advanced Master Mix were supplied by Fermentas (Pittsburgh, PA, USA) and Applied Biosystems (Thermo Fisher Scientific) respectively. A QuickChange II Site-Directed Mutagenesis Kit was purchased from Agilent Technologies-Stratagene (Santa Clara, CA, USA). Pyrrolidine dithiocarbamate (PDTC) was purchased from Abcam (Cambridge, UK). Cell counting kit-8 (CCK-8) reagent was purchased from Dojindo Laboratories (Kumamoto, Japan). Cell culture medium of RPMI-1640 and fetal bovine serum (FBS) were from Gibco (Thermo Fisher Scientific).

Cell culture

CNE1 and C666-1 as two human NPC cell lines, and the normal human nasopharyngeal epithelial cell line (NP69) were purchased from the American Type Culture Collection (ATCC, Manassas, USA). These cell lines were cultured in RPMI-1640 medium supplemented with 10% FBS, 50 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO_2 incubator.

Construction of NF-κB p65 overexpression plasmids

The overexpression plasmid of wild type (WT) NF-κB p65 (pcDNA3.1/p65^{WT}) was constructed by inserting the CDS of human p65 (GenBank: L19067.1) into the pcDNA3.1 vector. Additionally, the overexpression plasmid of mutant NF-κB p65 at the site of 310 (Lys→Arg), namely pcDNA3.1/p65^{K310R}, was generated with the QuickChange II Site-Directed Mutagenesis Kit according to the manufacturer's instructions. In order to perform immunoprecipitation to purify overexpressed p65 protein from NPC cells, His-tag was added to the p65 CDS to construct pcDNA3.1/His-p65^{WT} and pcDNA3.1/His-p65^{K310R}.

Construction of shRNA expression plasmids

The different shRNA sequences against human CBP mRNA (GenBank: U85962.3) were designed and constructed into the shRNA plasmid of pGCsi-U6/neo/GFP by Genkan (Shanghai, China). After identification, the most effective CBP shRNA expression plasmid (named shCBP) and a negative control shRNA expression plasmid (named shNC) were chosen for CBP knockdown experiments. The same method was used to construct and obtain an effective p300 shRNA expression plasmid (shp300, GenBank: NM_001429.3).

Cellular transfection

NPC cell lines were transfected with plasmids by Lipofectamine 2000 according to the manufacturer's instructions. Briefly, 3 µg of plasmids were mixed with 250 µl of serum-free medium, and

then 9 µl of Lipofectamine 2000 mixed with 250 µl of serum-free medium was added into the first 250 µl and incubated for 20 min at room temperature. Next, the 500 µl mixture of plasmids and Lipofectamine 2000 was added to each well of a six-well plate and incubated for 6 hours at 37°C in a 5% CO_2 incubator. Finally, the medium was replaced with serum containing medium 6 h later and incubated continually at 37°C in a 5% CO_2 incubator.

Co-immunoprecipitation experiment

The co-immunoprecipitation experiment was done according to the published procedure [20].

RNA extraction and real-time PCR

Total RNA in cells was isolated using Trizol reagent and used to synthesize cDNA with the RevertAid First Strand cDNA Synthesis Kit. Real-time PCR was then performed with the TaqMan Fast Advanced Master Mix on a 7500 Real-time PCR system (ABI, Foster, CA, USA) to determine the mRNA levels of target genes. At the same time, the β-actin gene was used as an internal control. PCR conditions were as follows: 50°C, 2 min; 95°C, 20 s and 40 cycles of 95°C, 3 s and 60°C, 30 s. The relative levels of gene expression were obtained with the formula: $2^{-\Delta\Delta CT}$ [37, 38].

Western blot analysis

Cellular lysate of each sample was centrifuged at 15,000 g for 10 min at 4°C to remove all insoluble materials. 40 µg of protein from each sample was subjected to 4–20% ExpressPlus PAGE Gel (Genscript, Nanjing, China) for electrophoresis, and then the protein was transferred onto PVDF membranes with the Mini-Protean System (Bio-Rad, Hercules, CA, USA). After blocking with 5% skim milk in TBST at room temperature (RT) for 1 h, the membranes were incubated with the different antibodies at 4°C overnight, and then incubated with HRP-conjugated anti-rabbit IgG or anti-mouse IgG at RT for 1 h. Finally, the bands on the membranes were visualized with electrochemiluminescence (ECL) after washing.

CCK-8 assay

Cells were seeded into 96-well cell culture plates at a density of 1×10^4 per well. After different treatments, cells were cultured for 45 h and then incubated with CCK-8 for the final 3 h. The OD value was detected at the absorbance of 450 nm [39, 40].

Statistical analysis

The statistical significance of differences between groups was evaluated by SPSS 19.0 software using one-way ANOVA with simultaneous multiple comparison with the Bonferroni method or unpaired *t* test. A *p*-value less than 0.05 was considered to be statistically significant.

Results

Acetylation of NF-κB p65 contributes to the proliferation of NPC cells

We observed that both NPC cell lines CNE1 and C666-1 exhibited markedly enhanced acetylation (Lys310) and phosphorylation (Ser536) of NF-κB p65 compared with the control NP69 cell line (Figures 1 A, B). In order to clarify the role of NF-κB p65 acetylation in the proliferation of human NPC cells, we constructed the NF-κB p65 expression plasmid with mutation of the acetylation site (Lys310). A further functional experiment demonstrated that over-expression of mutant NF-κB p65 reduced proliferation of human NPC cells compared to over-expression of wild type NF-κB p65 (Figures 1 C, D). Meanwhile, over-expression of mutant NF-κB p65 markedly reduced the phosphorylation of p65 (Figures 1 E, F). Taken together, these data indicate that the acetylation of NF-κB p65 might contribute to the proliferation of NPC cells through regulating p65 phosphorylation.

Interaction of CBP with NF-κB p65 is increased in NPC cells

Since, we found that the acetylation of NF-κB p65 was involved in the proliferation of NPC cells, in order to explore the potential mechanism of the formation of NF-κB p65 acetylation within NPC cells, the mRNA expression of four common acetyl transferases (CBP, p300, PCAF and GCN5) was detected in CNE1, C666-1 and NP69 cell lines. We observed that the mRNA expression levels of p300 and CBP genes were markedly increased in both CNE1 and C666-1 cell lines compared with the NP69 cell line (Figure 2 A). Notably, a further experiment showed that the interaction of p65 with CBP but not p300 at the protein level was markedly enhanced in both CNE1 and C666-1 cell lines relative to the NP69 cell line (Figure 2 B), suggesting that the up-regulation of CBP might promote the acetylation of NF-κB p65 in human NPC cells.

CBP expression is required for NF-κB p65 acetylation in NPC cells

To further determine the regulatory roles of CBP in NF-κB p65 acetylation and phosphorylation, a CBP knockdown experiment was performed in both CNE1 and C666-1 cell lines. The results showed that CBP knockdown with shCBP not only suppressed the acetylation of NF-κB

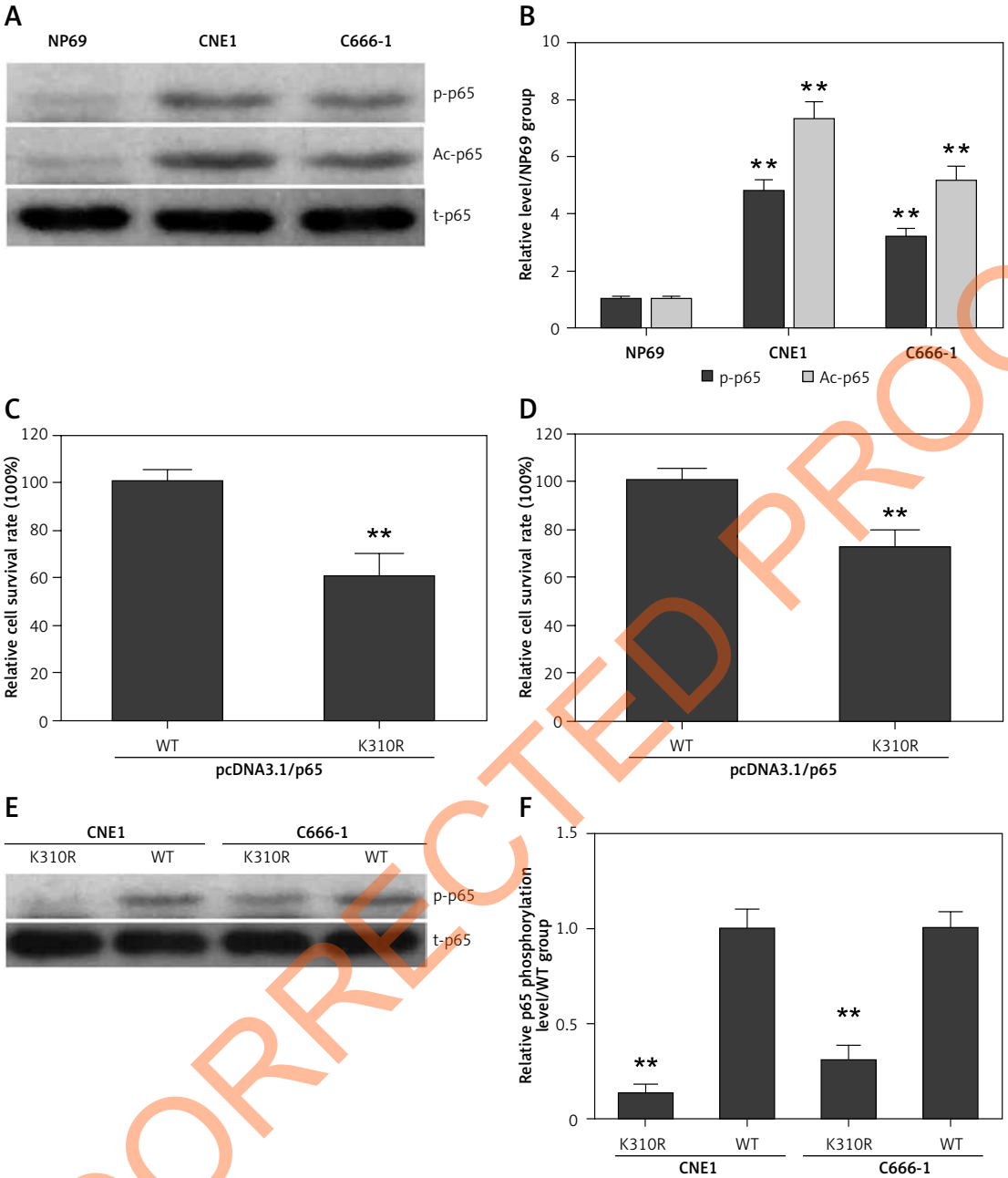

Figure 1. NF-κB p65 phosphorylation and acetylation in NPC cells. **A**, **B** – The phosphorylation and acetylation levels of NF-κB p65 in CNE1, C666-1 and NP69 cell lines were detected by Western blot analysis. $^{**}P < 0.01$ vs. NP69 group. **C**, **D** – The expression plasmids of WT p65 and mutant p65 (namely pcDNA3.1/p65^{WT} and pcDNA3.1/p65^{K310R}) were respectively transfected into both CNE1 (**C**) and C666-1 (**D**) cell lines, and then the proliferation of cells was examined by CCK-8 assay. $^{**}P < 0.01$ vs. pcDNA3.1/p65^{WT} group. **E**, **F** – The plasmids of pcDNA3.1/His-p65^{WT} and pcDNA3.1/His-p65^{K310R} were respectively transfected into both CNE1 (**E**) and C666-1 (**F**) cell lines, and then immunoprecipitation was done with anti-His-tag antibody. Finally, the phosphorylation of p65 was detected by Western blot in immunoprecipitated protein. $^{**}P < 0.01$ vs. pcDNA3.1/His-p65^{WT} group. The data are from one experiment, representative of three independent experiments. Results were represented as means ± SD (n = 3 in each group)

p65, but also reduced the phosphorylation of NF-κB p65 in CNE1 (Figures 3 A, B) and C666-1 (Figures 3 C, D) cell lines. Notably, although we could not observe the binding of p300 to p65 (Figure 2 B), we still performed p300 knockdown experiments to check the possible effect of p300 upregulation on p65 acetylation (Figure 4). The results showed that p300 knockdown had no significant effect on p65 acetylation in both NPC cell lines, indicating that p300 could not regulate p65 acetylation in NPC cells. Together, these data indicate that CBP expression is needed for the acetylation and further phosphorylation of NF-κB p65 in NPC cells.

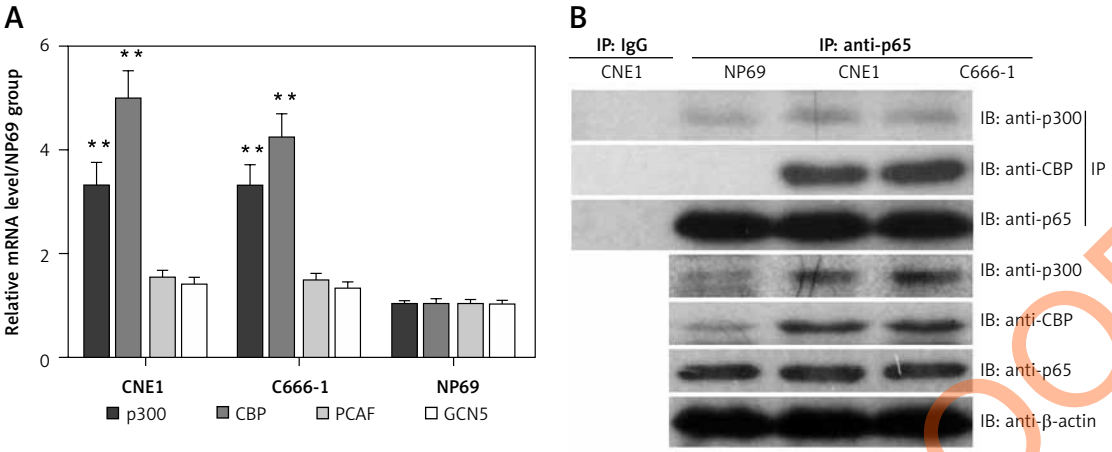

Figure 2. Binding of NF-κB p65 to p300 in NPC cells. **A** – mRNA expression levels of CBP, p300, PCAF and GCN5 in CNE1, C666-1 and NP69 cell lines were detected by real-time PCR. $^{**}P < 0.01$ vs. NP69 group. **B** – Interaction of p65 with CBP or p300 at the protein level in both CNE1 and C666-1 cell lines was determined by co-IP experiment. The data are from one experiment, representative of three independent experiments. Results were represented as means ± SD ($n = 3$ in each group)

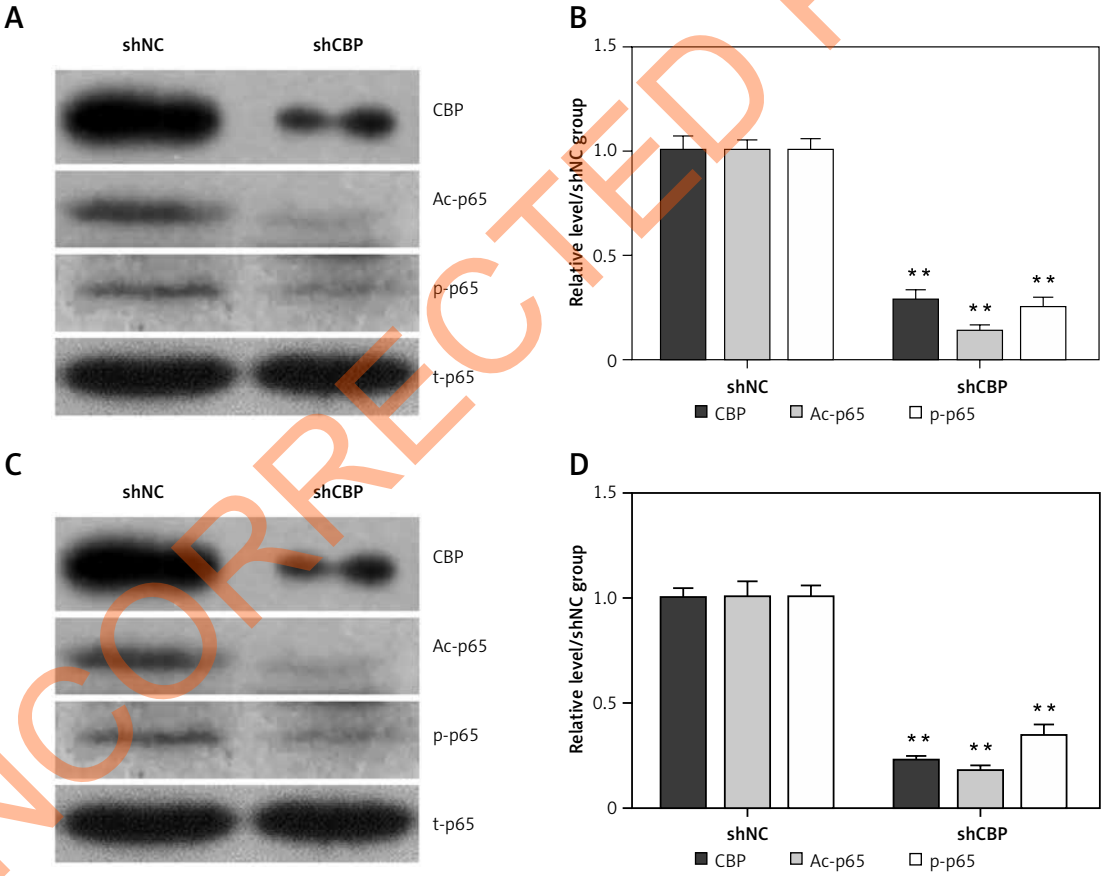

Figure 3. The roles of CBP in NF-κB p65 acetylation and phosphorylation in NPC cells. The NPC cell lines CNE1 (**A, B**) and C666-1 (**C, D**) were transfected with shRNA expression plasmids (shCBP or shNC) for 48 h, and the acetylation and phosphorylation levels of NF-κB p65 were detected by Western blot assay. $^{**}P < 0.01$ vs. shNC group. The data are from one experiment, representative of three independent experiments. Results were represented as means ± SD ($n = 3$ in each group)

CBP-mediated NF-κB p65 acetylation promotes NPC cell proliferation

Since we found CBP was required for NF-κB p65 acetylation in NPC cells (Figure 3), we further studied the proliferative roles of CBP-mediated NF-κB p65 acetylation in NPC cells. Expectedly, we observed that either shCBP or NF-κB inhibitor could markedly inhibit cellular proliferation of

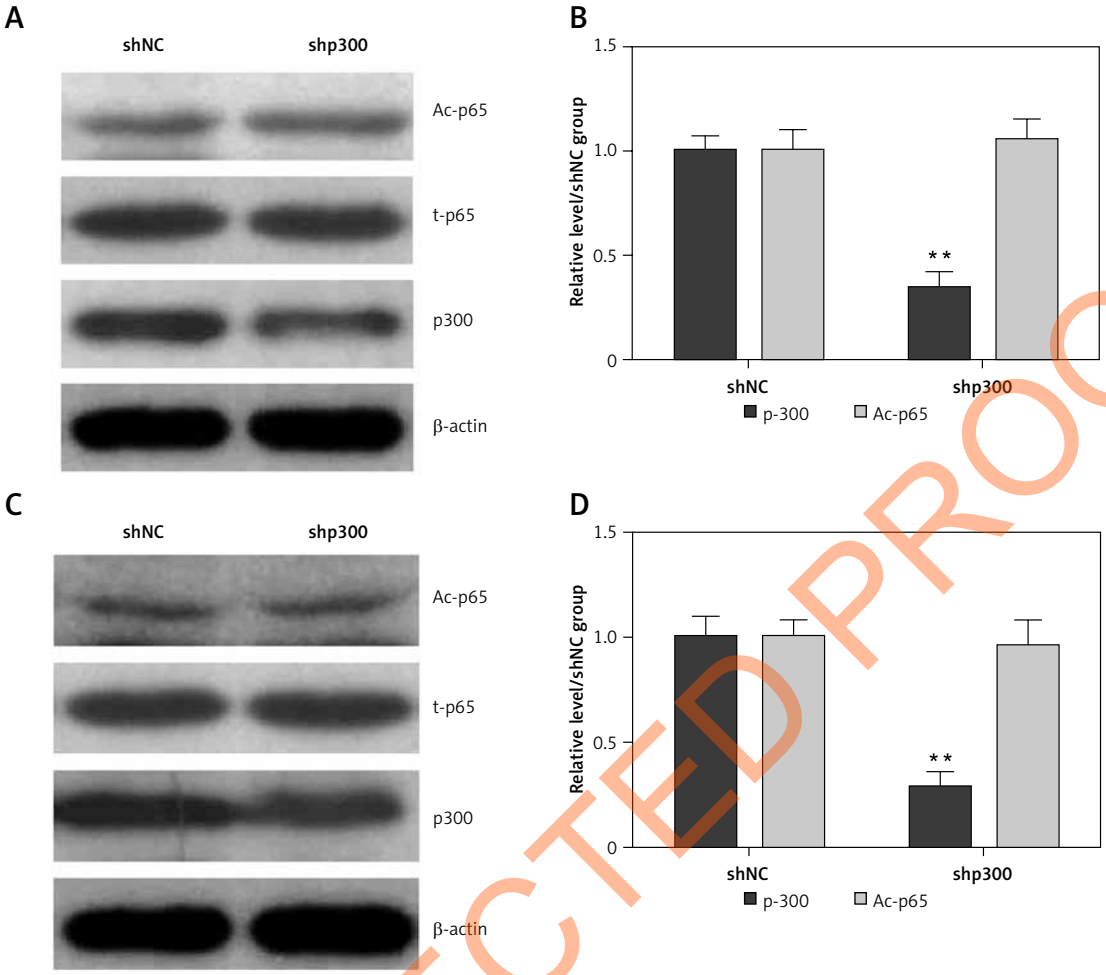

Figure 4. The effect of p300 on NF-κB p65 acetylation in NPC cells. The NPC cell lines CNE1 (**A, B**) and C666-1 (**C, D**) were transfected with shRNA expression plasmids (shp300 or shNC) for 48 h, and then the acetylation level of NF-κB p65 was detected by Western blot assay. The data are from one experiment, representative of three independent experiments. Results were represented as means ± SD (n = 3 in each group)

CNE1 and C666-1 cell lines (Figures 5 A, B). Additionally, to confirm the regulatory relationship between CBP and NF-κB p65, NF-κB p65 was overexpressed when CBP was silenced in both CNE1 and C666-1 cell lines. The results showed that the overexpression of NF-κB p65 markedly increased NPC cell proliferation, but CBP knockdown in NPC cells obviously inhibited the proliferative effect of NF-κB p65 overexpression (Figures 5 C, D). Together, these data indicate that CBP-mediated NF-κB p65 acetylation contributes to NPC cell proliferation.

Discussion

NPC is a malignant head and neck tumor occurring worldwide and especially common in Southeast Asia and China [41–43]. Notably, the molecular pathogenesis of NPC remains elusive. Increasing evidence has shown that the proliferation of cancer cells is regulated by a signaling pathway network [44–47]. Among these signaling pathways, the activation of NF-κB, especially the p65 subunit, is involved in the proliferation of NPC cells [48–50]; however, the molecular mechanism of the regulation of NF-κB p65 subunit activation remains largely unclear. In our current study, the phosphorylation and acetylation levels of NF-κB p65 were evaluated in human NPC cells. We observed that the phosphorylation and acetylation levels of NF-κB p65 were enhanced simultaneously in human NPC cells, indicating that acetylation might promote phosphorylation of NF-κB p65. In order to clarify the role of NF-κB p65 acetylation in the proliferation of human NPC cells, we constructed the NF-κB p65 expression plasmid with an acetylation site mutation. A further over-expression experiment demonstrated that the mutated NF-κB p65 reduced the proliferation of human NPC cells compared to wild type NF-κB p65.

Since NF-κB p65 acetylation was markedly enhanced and involved in the proliferation of NPC cells, we further explored the upstream regulation of NF-κB p65 acetylation. Reportedly, CBP, p300, PCAF and GCN5 have acetyl transferase activity and are involved in the regulation

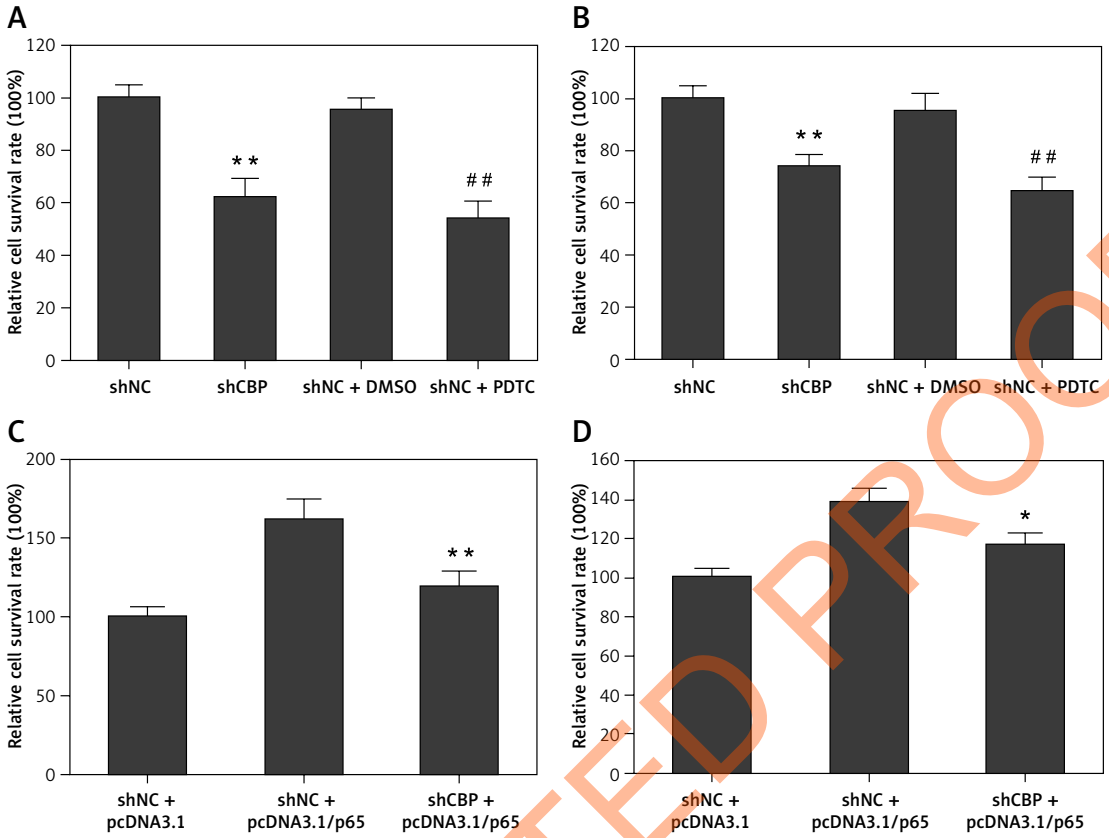

Figure 5. The roles of CBP-mediated NF-κB activation in proliferation of NPC cells. **A**, **B** – The NPC cell lines CNE1 (**A**) and C666-1 (**B**) were transfected with shRNA expression plasmids (shCBP or shNC), with or without NF-κB inhibitor PDTC treatment or DMSO as an inhibitor control for 48 h. Finally, cell proliferation was detected by CCK-8 assay. $^{**}P < 0.01$ vs. shNC group; $^{\#\#}p < 0.01$ vs. shNC + DMSO group. **C**, **D** – The NPC cell lines CNE1 (C) and C666-1 (**D**) were transfected with a shRNA expression plasmid (shCBP or shNC) together with the pcDNA3.1/p65^{WT} or pcDNA3.1 vector for 48 h, then cell proliferation was detected by CCK-8 assay. $^{*}P < 0.05$, $^{**}p < 0.01$ vs. shNC + pcDNA3.1/p65^{WT} group. The data are from one experiment, representative of three independent experiments. Results were represented as means ± SD ($n = 3$ in each group)

of transcription factors and signaling molecules [30, 51–54]. Therefore, the expression levels of CBP, p300, PCAF and GCN5 were further examined in human NPC cells, and we found that the expression levels of p300 and CBP but not PCAF and GCN5 were markedly enhanced in human NPC cells. A further experiment demonstrated that the physical interaction of NF-κB p65 with CBP at the protein level was enhanced in human NPC cells. Notably, although relatively weak binding of NF-κB p65 to p300 could also be detected, there was no significant increase of their binding in NPC cells. So, we focused on studying the roles of CBP, and found that CBP silencing could not only suppress the acetylation and phosphorylation of NF-κB p65 but also reduce the cellular proliferation of human NPC cells. These data indicate that CBP-mediated NF-κB p65 acetylation is essential for its phosphorylation and cellular proliferation in human NPC cells.

It is worth mentioning that Cai *et al.* reported that PCAF-dependent STAT3 acetylation promotes the proliferation of human NPC cells [55]. In their study, PCAF expression was up-regulated in human NPC cells in response to C5a stimulation. In contrast, we detected the expression of p300, CBP, PCAF and GCN5 in human NPC cells without C5a stimulation. That can explain why PCAF expression was not increased in human NPC cells in our present study. Additionally, Liao *et al.* reported that high expression of p300 is linked to aggressive features and poor prognosis of NPC [56]. In our current study, we observed higher expression of p300 in NPC cells than that in normal human nasopharyngeal epithelial cells; however, p300 knockdown had no significant effect on p65 acetylation, indicating that p300 could not regulate p65 acetylation in NPC cells. In addition, various types of posttranslational modification lead to extremely flexible regulation of protein function. Since NF-κB activation can be regulated by phosphorylation, ubiquitination, acetylation and methylation [57–59], further research needs to be performed to explore the potential interaction of these different types of posttranslational modification in human NPC cells.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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