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Original Article

p65 down regulates PKR through suppressing the activity of p53 in HeLa cell lines

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Abstract: As a major component of the innate immune response to viral infection, protein kinase R (PKR) is an interferon (IFN)-induced serine/threonine kinase that is ubiquitously expressed in all cells at low levels and is a key point of host defense to induce cell death in response to virus infection and tumorigenesis. The aim of the present study was to evaluate the role and mechanism of p65 in modulating PKR in HeLa cell lines. There was a low expression of PKR but high expression of p65 in HeLa cell lines. We found that knockdown of p65 increased the activity of p53 and autophosphorylation of PKR markedly and inhibited the proliferation, migration and invasion of HeLa cell lines. The increased expression and autophosphorylation of PKR induced by p65 knockdown could be suppressed by p53 inhibitor in HeLa cell lines. These results indicate that p65 down regulates PKR through suppressing the activity of p53 and promotes proliferation, migration and invasion of HeLa cell lines, and therefore may provide novel insights for control of human papillomavirus (HPV) infection and future development of new therapeutic strategies for cervical cancer.

Keywords: p65, PKR, p53, HeLa cell lines, proliferation, invasion

Introduction

Cervical cancer is the third most commonly diagnosed cancer worldwide and the fourth leading cause of cancer-related mortality worldwide, accounting for 10-15% of cancer-related mortalities [1]. High-risk human papillomavirus (HR-HPV) is considered to be the main etiological cause for cervical cancer [2]. As a major component of the innate immune response to viral infection, protein kinase R (PKR) is an interferon (IFN)-induced serine/threonine kinase that is ubiquitously expressed in all cells at low levels. Activated by binding of double-stranded (ds) RNA produced during virus replication, PKR phosphorylates its natural substrate, the alpha subunit of eukaryotic initiation factor 2 (eIF2) on serine residue 51, which inhibits translational initiation and also regulates activation of a wide range of factors including signal transducer and activator of transcription (STAT), interferon regulatory factor 1 (IRF-1), p53, JNK, p38 and NF-κB [3-7]. The balance between apoptosis and autophagy induced by PKR affects the effector cells of innate and adaptive immunity that mediate the inflammatory response [8-10]. Besides these, active PKR can also physically interact with inflammasome to trigger the release of proinflammatory cytokines, as well as stimulate programmed inflammatory cell death called pyroptosis [11, 12]. Therefore PKR functions as a transcriptional regulator exerting antiviral and antitumor activities [13] and as an inducer of apoptosis [14]. Nevertheless PKR-induced apoptosis could be remarkably decreased by inactivation of NF-κB through different mechanisms.

p65 is one of two major functional subunits in NF-κB family including NF-κB1 (p50/p105), NF-κB2 (p52/p100), relA (p65), relB, and c-Rel [15]. The p65-p50 heterodimers play an important role in the regulation of immune responses, embryo and cell lineage development, apoptosis, cell cycle progression, inflammation, and oncogenesis [16]. It is reported that there was high expression of p65 in cervical cancer infected with HPV [17, 18]. p53 is an important tumor suppressor protein, which induces a significant

expression of PKR by acting on the newly identified interferon-stimulated response element (ISRE), resulting in translational inhibition and cell apoptosis [19]. However, p53 can be inhibited not only by HR-HPV-encoded E6 protein but also p65. p65 can inhibit p53 through various mechanisms in many cells [20-24]. But there are no reports in HeLa cell lines.

The aim of this work was to elucidate whether p65 could act as an inhibitor of p53 to down regulate PKR autophosphorylation in HeLa cell lines. We investigated the activity of p53 with/without p65 knockdown and the PKR autophosphorylation with/without cyclic pifithrin-α in p65 knockdown HeLa cell lines. We also studied whether p65 could regulate the biological behavior of HeLa cell lines. Our results will provide a good proof elucidating the mechanism how PKR is inhibited by p65 in HeLa cell lines and provide a promising target for developing differential therapeutic strategies for cervical cancer.

Materials and methods

Reagents

The antibodies of p65 (C-20), p53 (FL-393), PKR (K-17), p-PKR (Thr 446), p-eIF2α (Ser 51), GAPDH (FL-335) and HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotech (Shanghai, China). Reagents for qRT-PCR and Western blotting were purchased from Life Technologies (CA, USA). Cyclic pifithrin-α (sc-222177) was purchased from Santa Cruz Biotech (Shanghai, China).

Cell culture

HeLa cell lines and human cervical keratinocytes were preserved in our laboratory (Central Laboratory of Tianjin First Central Hospital, Tianjin, China) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, CA, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies, CA, USA) and 100 U/mL penicillin G, 100 U/mL streptomycin sulfate at 37°C with 5% CO_2. The medium was changed in every 3 days. The cells were split when they reached 80% confluence.

shRNA transfection

The short hairpin RNA (shRNA) sequence targeting human p65 and shRNA non-targeting control (shNC) sequence were purchased from Santa Cruz Biotechnology. The sequence of p65 shRNA is: 5'-GATCCGCCCTATCCCTTTACGTCATTCAAGAGATGACGTAAAGGGATAGGGCTTTTTTGGAAA-3', and the sequence of NC shRNA is: 5'-GATCCTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTTTGGAAA-3'. Both shRNAs were synthesized and cloned into pSilencer vectors, respectively. HeLa cell lines were grown to 80% confluence in 6-well plates and then transfected with the vectors using Lipofectamine 2000 transfection reagent (Life Technologies, CA, USA). 48 h post transfection, HeLa cell lines were pelleted.

Overexpression of p53 in HeLa cell lines

The plasmid for p53 overexpression (pEGFPC1-p53) was preserved in our laboratory (Central Laboratory of Tianjin First Central Hospital, Tianjin, China). The empty vector pEGFPC1 was used as negative control. HeLa cell lines were transfected using Lipofectamine 2000 transfection reagent (Life Technologies, CA, USA). 48 h post transfection, HeLa cell lines were pelleted.

p53 activity assay

Nuclear extracts were prepared from HeLa cell lines using the EpiQuik Nuclear Extraction Kit (Amyjet Scientific Inc, Wuhan, China). Protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, IL, USA). The sequence-specific DNA-binding activity of p53 was determined using TF-Detect Human p53 activity assay kit (GeneCopoeia, Rockville, MD, USA) according to the manufacturer's protocol.

Quantitative real time RT-PCR (qRT-PCR)

Total RNA was extracted from the HeLa cell lines with Trizol reagents. The cDNA was synthesized using a reverse transcription kit. The primers for NF-κB p65, forward: TACCCTGAGGCTATAACTC; reverse: GACACTTGATAAGGCTTTG. PKR, forward: AAGAAGAGGCGAGAAACTAG; reverse: TTCAGAAGGGCTCTAACATG. p53, forward: AGAATCTCCGCAAGAAAGG; reverse: GCTGGTATGTCCTACTCCC. The PCR reaction was carried out for 40 cycles, which consisted of a pre-soak for 4 min at 94°C, denaturing for 30 s at 94°C, annealing for 30 s at 54-58°C, and extension for 6 min at 72°C after completion of the cycle. The results were calculated with the $2^{-\Delta\Delta Ct}$ method.

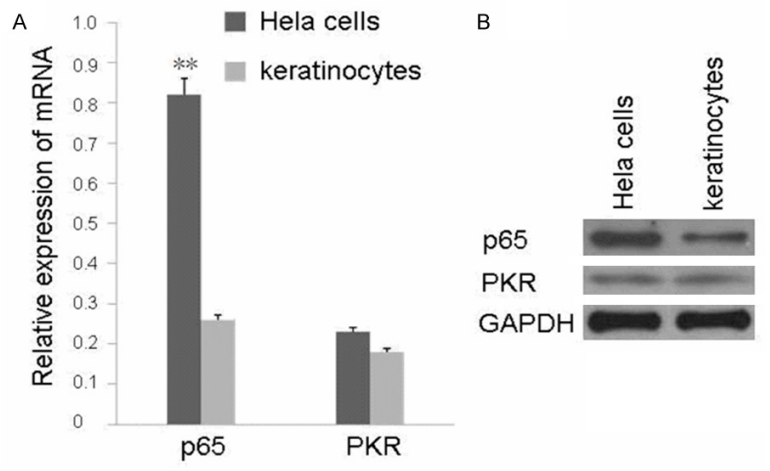

Figure 1. The high level of p65 and low level of PKR of HeLa cells, mRNA levels of p65 and PKR (A), protein levels of p65 and PKR (B) (n=3, mean ± SD. *P<0.05, **P<0.01, compared with the group of human cervical keratinocytes).

Western blotting

HeLa cell lines were collected and prepared for western blot. Whole-cell protein extracted was prepared and quantified by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Equal volume of proteins were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane (Millipore, MA, USA). The membrane was blocked with 5% skim milk for 30 min, incubated with primary antibodies (100-500 ng/mL) at 4°C overnight. Then membranes were washed by Tris-Buffered Saline Tween (TBST) for three times, followed by incubating with HRP-conjugated secondary antibodies at room temperature for 1 h. After washed with TBST for three times again, the immune complexes in the membranes were developed with ECL. The results were recorded with X-ray films in a dark room.

3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay

HeLa cell lines in different groups were seeded in 96 well plates at a density of 5,000 cells per well and cultured at 37°C in 5% CO_2 for 12, 24, 48, or 72 h. 25 μL of 0.5 mg/mL MTT solution purchased from Sigma Aldrich (St. Louis, MO, USA) was added and incubated for 4 h. After the medium was removed, formazan crystals generated by the cellular reduction activity were dissolved in 100 μL DMSO (Sigma Aldrich, St. Louis, MO, USA). Absorbance was measured at 570 nm with a Bio-Rad 400 microplate reader (Bio-Rad, USA).

Migration and invasion assay

The migration ability of HeLa cell lines was assayed by using the 24-well transwell chambers (Costar; New York, NY; pore size, 8 μm). For invasion assay, the filters were pre-coated with Matrigel basement membrane matrix (BD Biosciences, USA) for 4 h at 37°C. Procedures were similar for the migration and invasion assays. Transfected cells (each with 2×10^4 cells in 0.2 mL of serum-free DMEM medium) were added to the top chambers, while 500 μL of DMEM medium containing 15% FBS was added to the bottom chambers. Cells were incubated in a 5% CO_2 and 37°C incubator for 24 h. Then the non-invading cells and gel on the upper side of filters were removed with cotton-tipped swabs and the remaining cells on the underside of the filters were fixed with methanol for 30 min, were stained with 0.01% Eosin Stain, and were examined and counted under a microscope.

Statistics

Experimental results are shown as the mean ± SD. The statistical analysis was performed using SPSS19.0 statistical software. *P* values were determined by Student's *t*-test or two-way analysis of variance (ANOVA). Significance was defined as *P<0.05, **P<0.01.

Results

Expression of p65 and PKR in HeLa cell lines

Firstly, we sought to validate whether p65 could inhibit the expression of PKR. To address this, we evaluated the expression of p65 and PKR in HeLa cell lines or human cervical keratinocytes. As shown in **Figure 1A** and **1B**, we demonstrated that there was a significantly higher expression of p65 in HeLa cell lines as compared to human cervical keratinocytes.

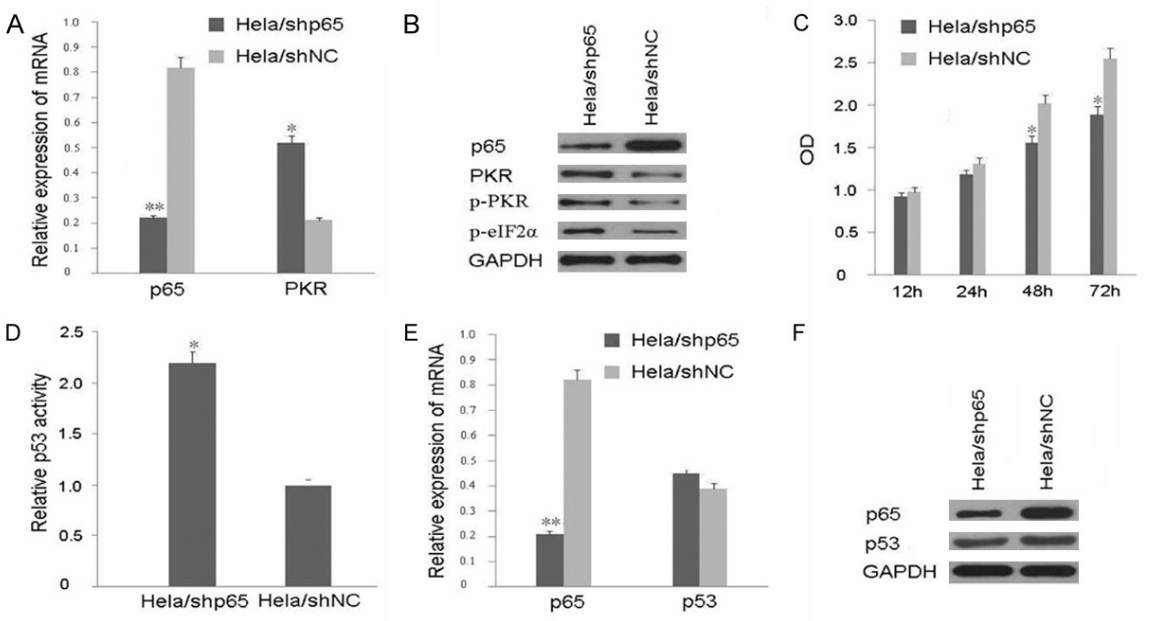

Figure 2. The knockdown of p65 increases mRNAs levels of p65 and PKR (A), protein levels of p65, PKR, p-PKR, p-eIF2α (B), and suppresses the proliferation of HeLa cells at 48 h after transfection (C), and enhances the activity of p53 in HeLa cells (D), mRNA levels of p65 and p53 in HeLa cells (E), proteins levels of p65 and p53 in HeLa cells (F). HeLa/shp65: HeLa cells transfected with pSilencer-shRNA-p65 plasmid. HeLa/shNC: HeLa cells transfected with pSilencer-shRNA-NC plasmid (n=3, mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$, compared with the group of human cervical keratinocytes).

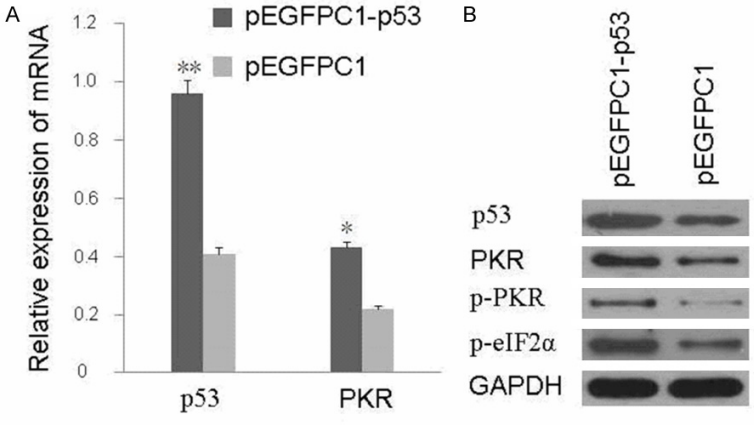

Figure 3. p53 induces a significant expression and autophosphorylation of PKR in HeLa cell lines, mRNA levels of p53 and PKR in HeLa cells (A), protein levels of p53, PKR, p-PKR, p-eIF2α in HeLa cells (B) (n=3, mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$).

Knockdown of p65 increases the expression and autophosphorylation of PKR and enhances the activity of p53 in HeLa cell lines

Whether p65 could inhibit the expression and autophosphorylation of PKR in HeLa cell lines was confirmed by p65 gene knockdown HeLa cell lines. Our results showed markedly higher levels of PKR and increased phosphorylation levels of eIF2α which was the downstream substrate of PKR in p65 gene knockdown HeLa cell lines (**Figure 2A** and **2B**). Although the expression of p53 didn't change distinctly by p65 knockdown compared to pSilencer-shNC plasmid transfected group, the transcriptional activity of p53 increased significantly in p65 knockdown HeLa cell lines as shown in **Figure 2D-F**. These results indicate that p65 down regulates the expression of PKR and suppresses the transcriptional activity of p53 in HeLa cell lines.

Knockdown of p65 suppresses the proliferation, migration and invasion of HeLa cell lines

The cell proliferation of HeLa cell lines was measured by MTT assay in vitro. As shown in **Figure 2C**, there was initially no difference in OD values between the p65 knockdown group and HeLa/shNC control group (12 and 24 h). However, at 48 and 72 h, knockdown of p65

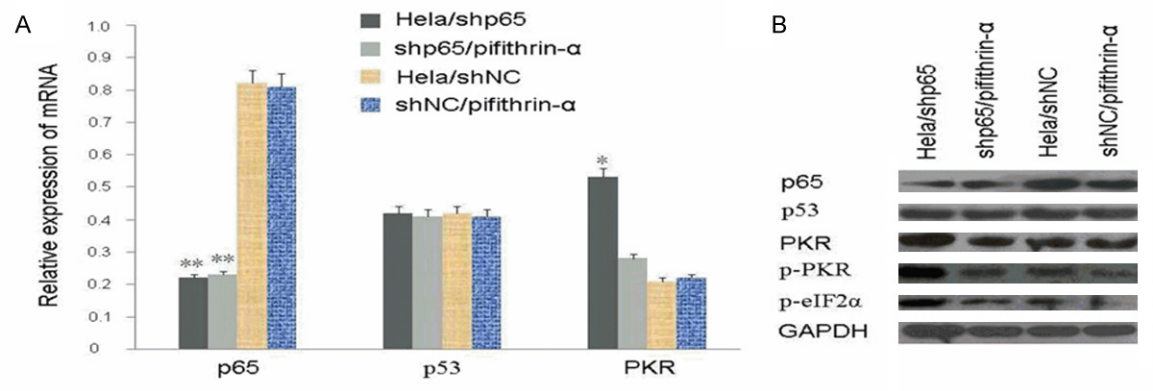

Figure 4. Cyclic pifithrin-α inhibits the increased expression and autophosphorylation of PKR induced by p65 knockdown, mRNA levels of p65, p53 and PKR in HeLa cells (A), protein levels of p65, p53, PKR, p-PKR and p-eIF2α in HeLa cells (B) (n=3, mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$).

gene markedly suppressed the proliferation of HeLa cell lines compared to HeLa/shNC control group (the OD value 1.56±0.23 vs. 2.02±0.34, $^{*}P<0.05$ and 1.89±0.29 vs. 2.55±0.41, $^{*}P<0.05$). Transwell detection was performed to evaluate the effect of p65 on migration and invasion of HeLa cell lines. The number of migrating cells was significantly reduced in p65 knockdown group compared to HeLa/shNC control group. Similarly, the number of invading cells was also reduced in p65 knockdown group compared to HeLa/shNC control group.

p53 induces a significant expression and autophosphorylation of PKR in HeLa cell lines

PKR is an important target gene of p53. To validate whether p53 is involved in the regulation of PKR in HeLa cell lines, we overexpressed the p53 gene in HeLa cell lines transfected with a p53 overexpression plasmid (pEGFPC1-p53). Indeed, as shown in **Figure 3A** and **3B** the overexpression of p53 induced a significant expression and autophosphorylation of PKR in HeLa cell lines in line with the previous report [19].

Cyclic pifithrin-α inhibits the increased expression and autophosphorylation of PKR induced by p65 knockdown

Based on these studies, we postulated that p65 might suppress the activity of p53 to down regulate PKR in HeLa cell lines. To validate the hypothesis, cyclic pifithrin-α, a p53 inhibitor was used to inhibit the transcriptional activity of p53 with to examine the effects of p65 knockdown on expression and autophosphorylation of PKR. Our results reveal that the increased expression and autophosphorylation of PKR induced by p65 knockdown is inhibited by cyclic pifithrin-α as shown in **Figure 4A** and **4B**.

Discussion

As discussed previously, HeLa cell is a kind of cervical cancer cell whose DNA is integrated with HPV18. It is reported that under hypoxic conditions, HR-HPV-encoded E6 protein promotes the activation of NF-κB (p65-p50 heterodimers) [17]. Another report has shown that HPV16 E6, E7, or E6/E7 can induce the expression of p65 and the binding of NF-κB DNA, as well as stimulate expression of several additional genes that are known to be involved in NF-κB activation in cervical keratinocytes [18]. Therefore, our findings indicate that HPV18 may promote the expression of p65 in HeLa cell lines in line with previous report. Several studies have reported that HPV gene products could modulate the expression, phosphorylation, activity and localization of PKR [25, 26]. However, whether p65 is involved in the regulation of PKR is not clear yet.

Pal et al. reported that NF-κB may cripple cellular p53 activities through various mechanisms [21]. Jeong et al. demonstrated that the p65 subunit of NF-κB was uniquely involved in p53 inhibition in human T-cell lymphotrophic virus type I (HTLV-I) transformed cells [24]. Our present study reveal the underlying mechanism that p65 interferes with the activity of p53 to down regulate PKR and enrich the existing

knowledge by showing p65 as a novel regulator of PKR in HeLa cell lines.

Though there is a high expression of p65, which is correlated with proliferation, migration and invasion of HeLa cell lines is not clear yet.

Therefore we investigated the effects of p65 expression on these aggressive biological behaviors of HeLa cell lines in vitro. The results show that the proliferation of HeLa cell lines is significantly suppressed by p65 knockdown compared with pSilencer-shNC plasmid transfected group indicating that p65 had a major impact on the proliferation of HeLa cell lines. The transwell assay demonstrates that the knockdown of p65 significantly decreases the migration and invasion capacity of HeLa cell lines. Consistent with our results, previous studies also reported that p65 is involved in cell migration and metastasis indicating that p65 may be a potential target to prevent cancer metastasis [27].

In summary, there is a low expression of PKR but high expression of p65 in HeLa cell lines and p65 down regulates PKR through suppressing the activity of p53 and promotes proliferation, migration and invasion of HeLa cell lines. Therefore future investigations will be directed to p65 antagonist in vivo to recover the activity of p53 and up regulate PKR, which may provide novel insights for control of HPV infection and future development of new therapeutic strategies for cervical cancer.

Disclosure of conflict of Interest

None.

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