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Original Article

Overexpression of yes-associated protein contributes to apoptosis of lung cancer

Limei Sun, Qingfu Zhang, Hongjiu Ren, Yitong Xu, Ailin Jin, Xueshan Qiu

Department of Pathology, The First Affiliated Hospital and College of Basic Medical Sciences of China Medical University, Shenyang, PR China

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Abstract: The Hippo signaling pathway was discovered as a key regulator of organ size and tumorigenesis. Yes-associated protein (YAP), a candidate human oncogene, is one of the two main downsream effectors of Hippo pathway. Dysregulation of the Hippo-YAP pathway plays an important role in various tumors. Here, we show that the Yap play a conserved role in cell apoptosis of non-small-cell lung cancer (NSCLC). We overexpressed or depleted YAP in NSCLC cell lines and found that YAP plays a dual role in NSCLC cell lines of different P53 phenotypes. These results identify YAP to be a novel regulator in NSCLC cell apoptosis and might be a new potential therapeutic target for NSCLC.

Keywords: Hippo signaling pathway, yes-associated protein, apoptosis, non-small-cell lung cancer

Introduction

Lung cancer is one of the major causes of cancer-related death worldwide, and Non-small cell lung cancer accounts for 80% of all lung cancer cases [1, 2]. Though progress made in past decades, conventional therapies may have reached a therapeutic bottleneck [3]. So it is vital to unveil the biological functions of the disease for the sake of useful biomarkers, and to explore novel therapeutic targets.

Yes-associated protein (YAP) is the homogenous gene of Yki which is found in Drosophila Hippo signal pathway, the human YAP gene is located on human chromosome bands 11q22 (common in several human tumors), encoding the 65 kD protein [4, 5]. YAP as a transcriptional coactivator combines with the TEAD family, P73, SMAD family (BMP pathway), Bric2, Bric5 (IAP family), cytoplasmic binding domain of ErbB4, PEBP2 α through its structure of the PPXY domain in cell growth [6-9]. YAP plays a key role in the regulation of cell proliferation, apoptosis and volume of organ. Recent studies have found that 20% to 30% human cancer cells contain increased levels of YAP protein including hepatocellular carcinoma (HCC), lung cancer, breast cancer, colorectal cancer, ovarian cancer [4, 5]. Transgenic mice with liver-specific YAP overexpression have increased 4 times in liver size and eventually led to tumor, YAP overexpression in MCF10A cells also promoted epithelial-mesenchymal transgenic (EMT) transition [10].

Despite the growing evidences of YAP as a crucial factor of human cancers, the effect of YAP on apoptosis is still controversial. In the present study, we overexpressed and knocked down YAP in NSCLC cell lines of different P53 phenotypes to investigate its dual effects on apoptosis.

Materials and methods

Cell lines

A549, H1299 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (Invitrogen), 100 IU/ml penicillin (Sigma, St. Louis, MO, USA), and 100 μg/ml streptomycin (Sigma). Cells were grown on sterile tissue culture dishes and were passaged every 2 days using 0.25% trypsin (Invitrogen).

Table 1. Prime sequences

Name	Prime sequences
β-Actin Forward	5'-CATGTACGTTGCTATCCAGGC-3'
β-Actin Reverse	5'-CTCCTTAATGTCACGCACGA-3'
YAP Forward	5'-CGCTCTTCAACGCCGTCA-3'
YAP Reverse	5'-AGTACTGGCCTGTCGGGAGT-3'
BAX Forward	5'-TCCACCAAGAAGCTGAGCGAG-3'
BAX Reverse	5'-GTCCAGCCCATGATGGTTCT-3'
P73 Forward	5'-TTCAACGAAGGACAGTCTGC-3'
P73 Reverse	5'-CAGGGTCATCCACATACTGC-3'

Plasmid and transfection

The plasmid of pcDNA3.1-hYAP was a kind gift from Dr Subham Basu (Cancer Research UK, London, UK). The plasmid of pOTB7-TP53 was purchased Proteintach (Proteintach, China) pcDNA3.1-hYAP or pOTB7-TP53 control or plasmid was transfected into cells using Attractene Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The mRNA level was assessed 48 h later by real-time PCR and protein level was assessed 72 h later by western blotting.

Small-interfering RNA experiment

YAP siRNA was purchased from Gene Pharma (Gene Pharma, China) Cells were transfected with siRNA (0.2 nM per six-well plate) using HiPerFect Transfection Reagent (Qiagen, Hilden, Germany) according to manufacturer's instructions. Following transfection, the mRNA level was assessed 48 h later by real-time PCR and protein level was assessed 72 h later by western blotting.

Quantitative real-time PCR (SYBR green method)

Quantitative real-time PCR was performed using SYBR Green PCR master mix (Applied Biosystems) in a total volume of 20 μl on 7900HT Fast Real-Time PCR System (Applied Biosystems) as follows: 95°C for 30 seconds, 40 cycles of 95°C for 5 seconds, 60°C for 30 seconds. A dissociation step was performed to generate a melting curve to confirm the specificity of the amplification. β-actin was used as the reference gene. The relative levels of gene expression were represented as ΔCt=Ct gene-Ct reference, and the fold change of gene expression was calculated by the 2-ΔΔCt method. The primers used were shown in **Table 1**. Experiments were repeated in triplicate.

Western blot analysis

Total proteins from cell lines were extracted in lysis buffer (Thermo Fisher Scientific, Rockford, IL) and quantified using the Bradford method. Fifty micrograms of protein were separated by SDS-PAGE (10%). After transferring, the polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) were incubated overnight at 4°C with the following rabbit polyclonal antibodies against YAP (1:500; Cell Signaling Technology, USA), goat polyclonal antibodies against P73 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), mouse polyclonal antibodies against BAX (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse polyclonal antibodies against P53 (1:500, Beyotime, China). After incubation with peroxidase-coupled anti-mouse/rabbit IgG (Santa Cruz Biotechnology) at 37°C for 2 hours, bound proteins were visualized using ECL (Thermo Fisher Scientific) and detected using BioImaging Systems (UVP Inc., Upland, CA, USA). The relative protein levels were calculated based on GAPDH as the loading control.

Apoptosis analysis

For detection of apoptosis, adherent cells were both collected and resuspended in cold PBS for analysis. Cells were stained with Annexin V-FITC Apoptosis Kit (BD Pharmingen, USA) to monitor apoptosis cells and propidium iodide (PI) to detect dead cells. Data were collected using BD systems.

Statistical analysis

SPSS version 19.0 was used for all analyses. The Student's t-test was used to compare other data. *p* value was based on the two-sided statistical analysis, and $P<0.05$ was considered to indicate statistical significance.

Results

YAP promotes apoptosis of lung cancer cells of P53 deletion type

We overexpressesd YAP in H1299 cell line as known P53-/-. Transfection of pcDNA3.1-hYAP plasmid obviously increased YAP mRNA and

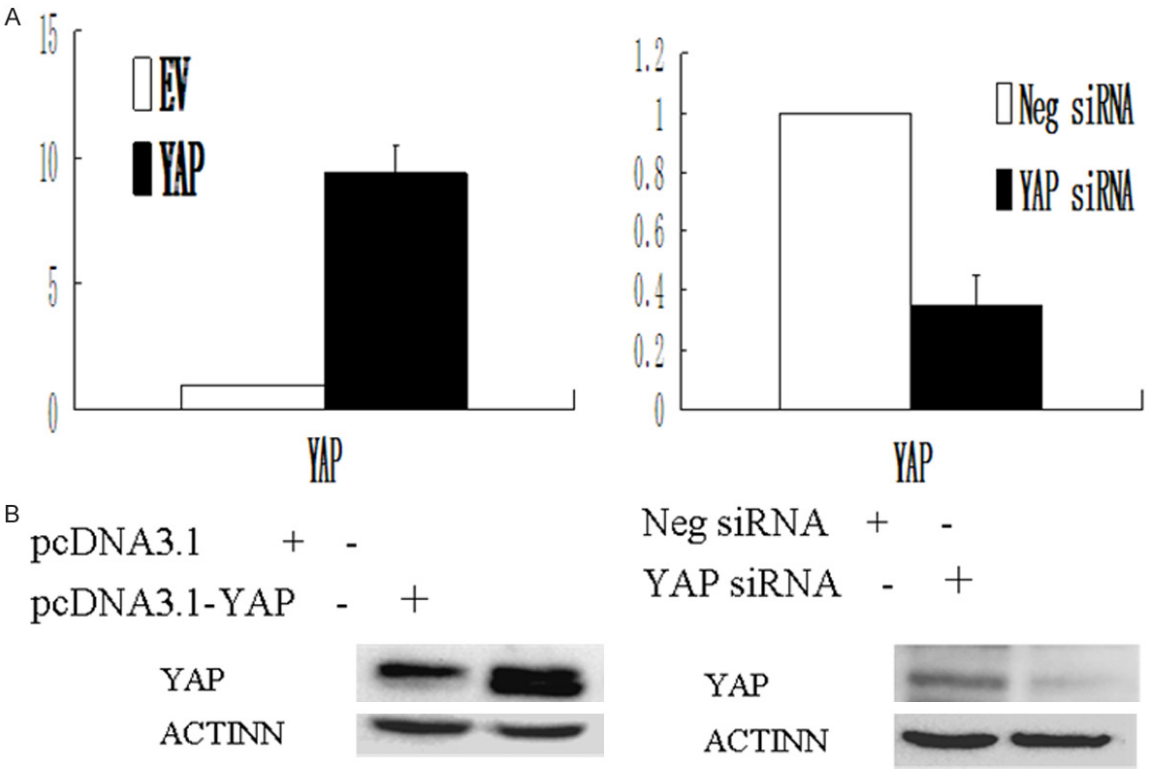

Figure 1. Yes-associated protein (YAP) expression in lung cancer cells transfected with YAP or YAP specific siRNA. (A) Real-time PCR; and (B) western blot analysis showed the effects of YAP overexpression and YAP silencing. Left, H1299 cells transfected with pcDNA3.1-hYAP and empty vector. Right, H1299 cells transfected with specific YAP siRNA and negative control siRNA.

protein levels compared with control empty vector (**Figure 1A**, **1B**). Clearly, a significant population of early apoptosis (H1299: 5.34%) was observed in H1299 cells with YAP overexpression compared with control empty vector (H1299: 8.85%) (**Figure 2A**).

We also knocked down YAP in H1299 cell line using YAP-specific siRNA. Efficient depletion of YAP expression was confirmed by real-time PCR and western blot analysis (**Figure 1A**, **1B**). YAP depletion reduced the early apoptosis in H1299 cells (H1299: 9.83%) compared with scramble controls (H1299: 6.96%) (**Figure 2B**).

YAP inhibits apoptosis of lung cancer cells of P53 wide type

To determine the effect of YAP on apoptosis in A549 cells with p53+/+, we overexpressed YAP in A549 cells. Transfection of pcDNA3.1-hYAP plasmid obviously increased YAP mRNA and protein levels compared with control empty vector (**Figure 3**). Clearly, YAP overexpression inhited apoptosis in A549 cells (B: 4.62%) compared with control empty vector (A: 7.93%) (**Figure 4**).

To further certificate that YAP inhibit cell apoptosis induced by P53, we transfect pOTB7-TP53 or pcDNA3.1-hYAP plasmid in H1299 cells, YAP and P53 protein levels were analyzed by western blot (**Figure 5A**). P53 overexpression promoted cell apoptosis (H1299: 4.36%) compared with control empty vector (H1299: 3.00%); overexpression of P53 and YAP reduced cell apoptosis (H1299: 1.99%) compared with only overexpressing P53 (**Figure 5B**). So we identify that YAP inhibit cell apoptosis induced by P53.

YAP regulates BAX depend on P73

To further explore the mechanisms, we examined the changes of mRNA and protein levels of BAX which was the common downstream apoptosis factor of P53 and P73. Both mRNA and protein levels of BAX increased after YAP overexpressing, and the results were opposite after YAP silencing (**Figure 6A**, **6B**, left and middle). However, up-regulated YAP expression significantly decreased both mRNA and protein levels of BAX in A549 cells (**Figure 6B**, right). All the results were consistent with

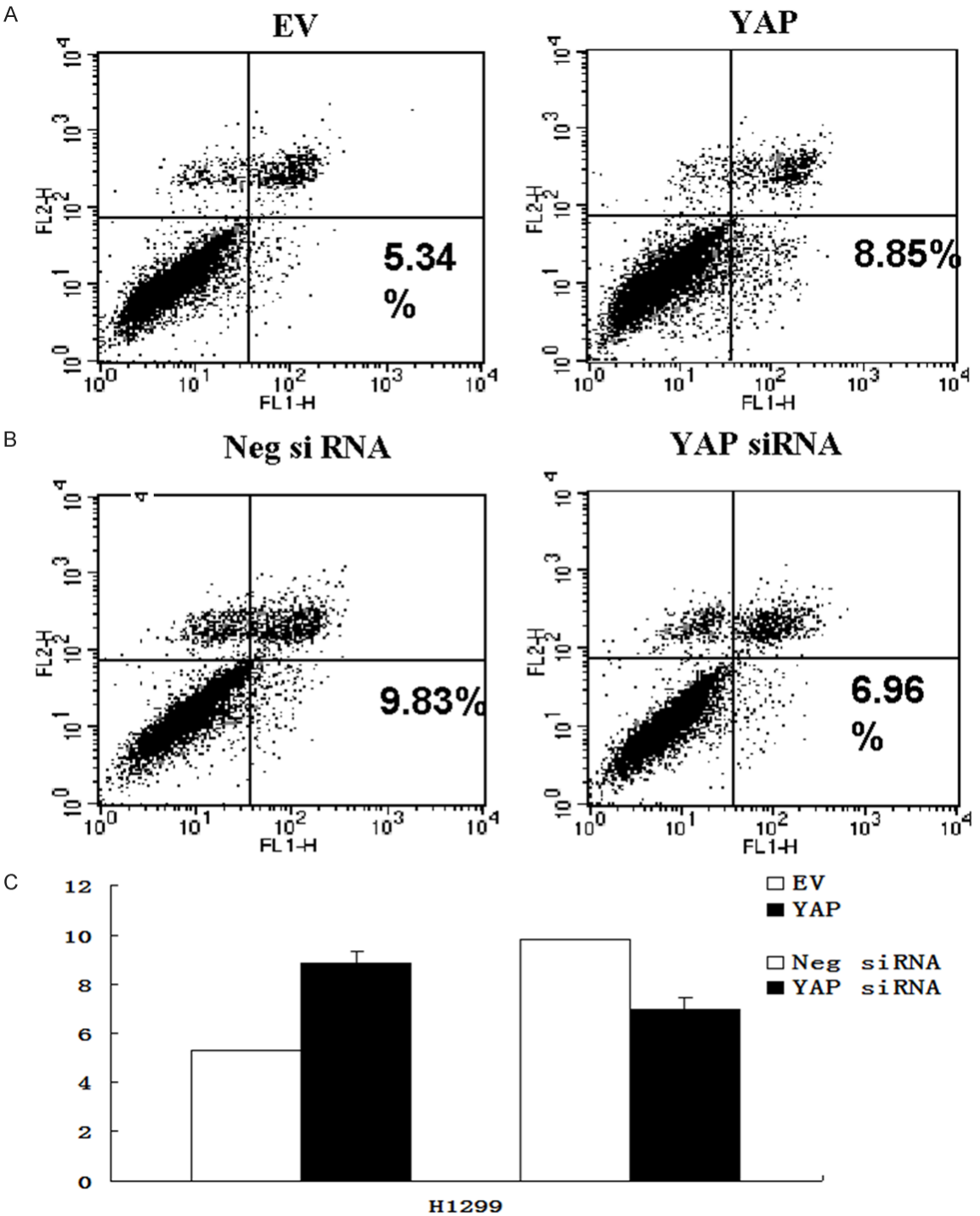

Figure 2. Yes-associated protein (YAP) expression in lung cancer cells transfected with YAP or YAP specific siRNA. serum-free, after 48 h, Cells were stained with Annexin V-FITC Apoptosis Kit. A. Overexpression YAP significantly promoted apoptosis in H1299 cells with P53 depletion. B. YAP down-regulation suppressed apoptosis. C. Columns, mean for apoptosis experiments; bars, SD; $P<0.05$.

apoptosis data. As shown in **Figure 7**, we observed the identical results after up-regulating YAP and P53 in H1299 cells, which was also consistent with apoptosis data. YAP

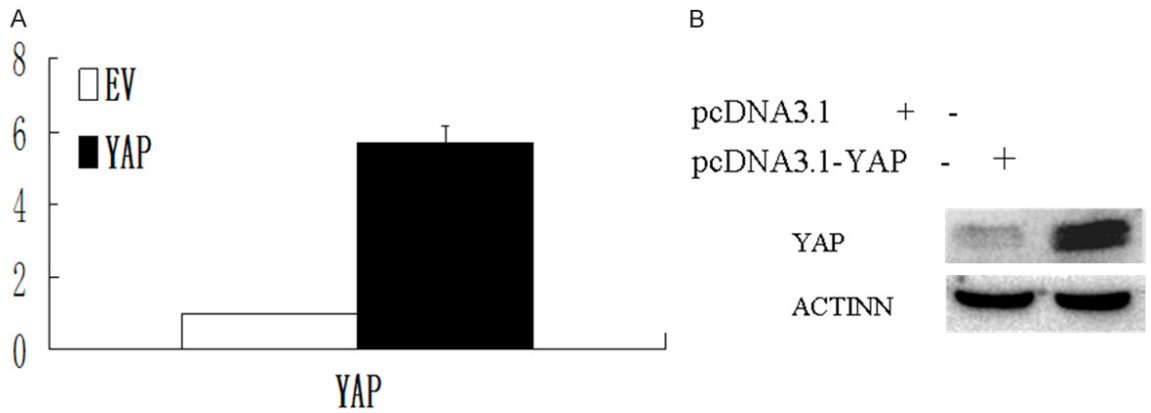

Figure 3. Yes-associated protein (YAP) expression in lung cancer cells transfected with YAP or YAP specific siRNA. (A) Real-time PCR; and (B) western blot analysis showed the effects of YAP overexpression and YAP silencing. A549 cells transfected with pcDNA3.1-hYAP and empty vector.

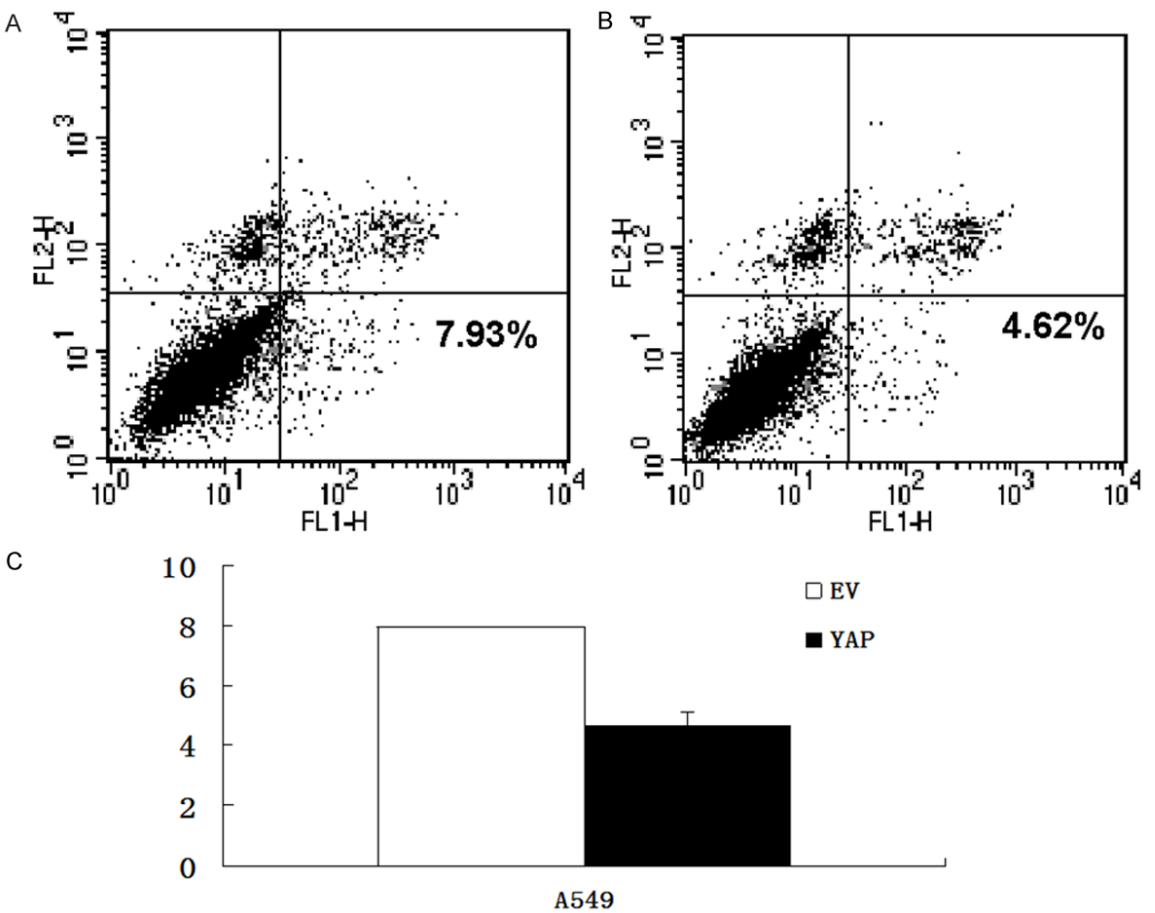

Figure 4. Yes-associated protein (YAP) expression in lung cancer cells transfected with YAP or empty vector. Serum-free, after 48 h, Cells were stained with Annexin V-FITC Apoptosis Kit. A. A549 cells apoptosis level when transfected with empty vector. B. Overexpression YAP significantly inhibited apoptosis in A549 cells with wild-type P53 depletion. C. Columns, mean for apoptosis experiments; bars, SD; $p<0.05$.

did not have DNA binding do domain, so we speculated it regulated BAX through P73. As shown in **Figure 8**, YAP interacted P73 after YAP overexpression in H1299 cells.

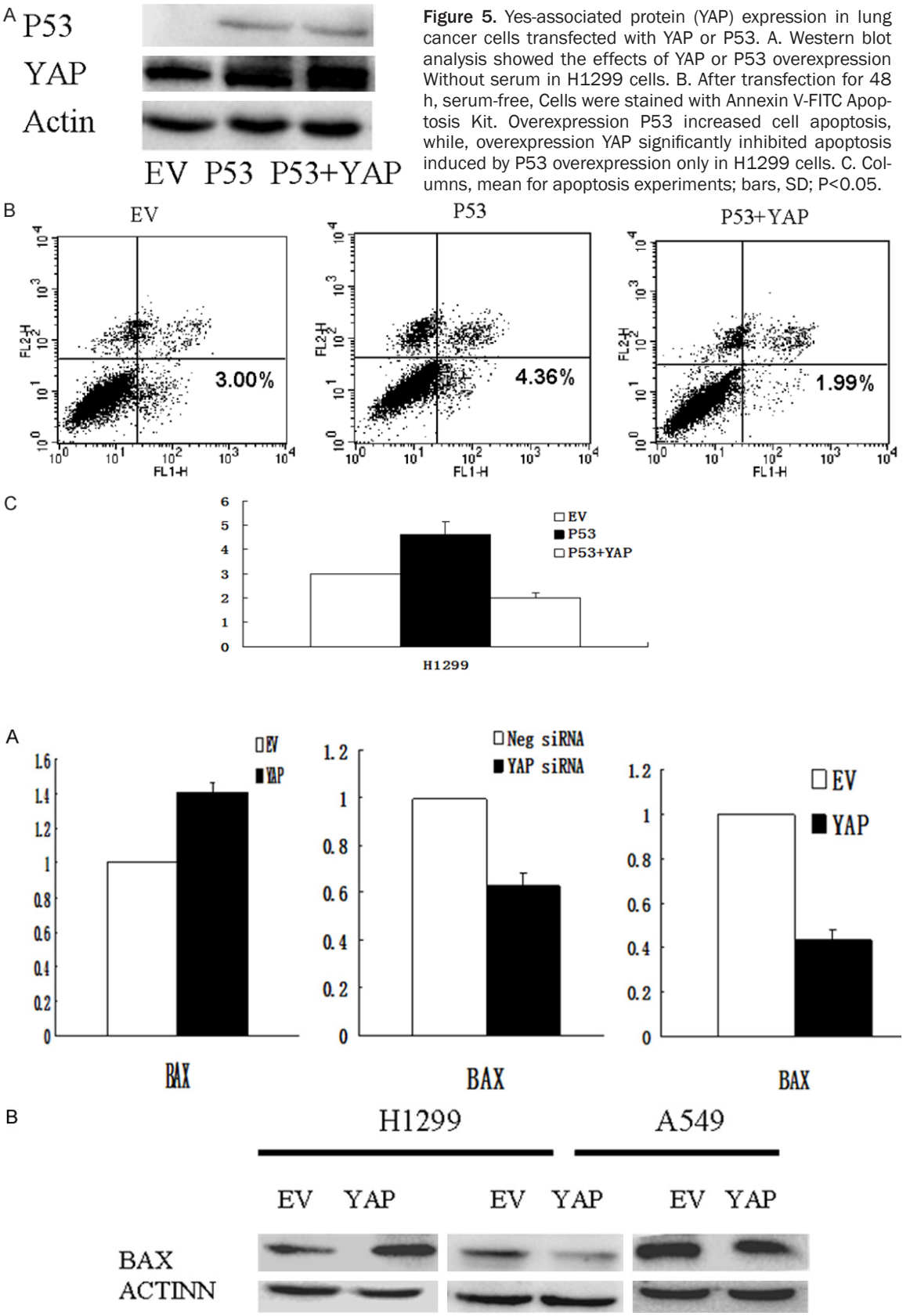

Figure 5. Yes-associated protein (YAP) expression in lung cancer cells transfected with YAP or P53. A. Western blot analysis showed the effects of YAP or P53 overexpression Without serum in H1299 cells. B. After transfection for 48 h, serum-free, Cells were stained with Annexin V-FITC Apoptosis Kit. Overexpression P53 increased cell apoptosis, while, overexpression YAP significantly inhibited apoptosis induced by P53 overexpression only in H1299 cells. C. Columns, mean for apoptosis experiments; bars, SD; P<0.05.

Figure 6. Yes-associated protein (YAP) expression in lung cancer cells transfected with YAP or YAP specific siRNA. A. Real-time PCR, Left and middle, H1299 cells , the changes of BAX expression after overexpression YAP or YAP

silencing; Right, A549 cells. B. Western blot analysis showed the expression of BAX after overexpression YAP or YAP silencing.

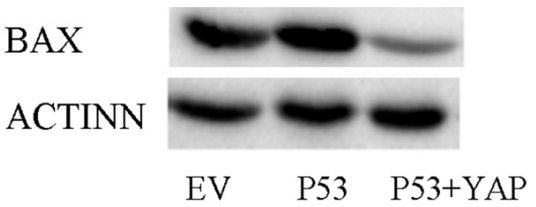

Figure 7. Yes-associated protein (YAP) expression in lung cancer cells transfected with YAP or P53. Western blot analysis showed the expression of BAX after overexpression YAP or P53 in H1299 cells.

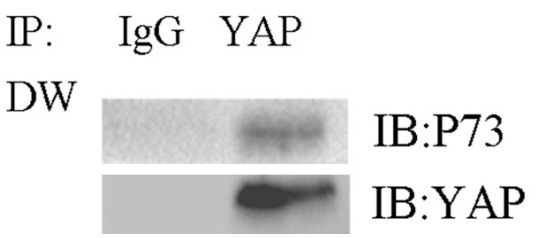

Figure 8. Yes-associated protein (YAP) expression in H1299 cells transfected with YAP, after 48 h, IP experiment showed YAP interacted P73.

Discussion

Overexpression of YAP has been involved in tumor progression in various human cancers, However, the effect of YAP on apoptosis is still controversial. In this study, we demonstrated that overexpression of YAP had a dual effect on cell apoptosis, and its function was associated with phenotype of P53 in NSCLC.

In previous study, YAP expression and function was elevated in hepatocellular carcinoma, prostate cancer, colon cancer, lung cancer and breast cancer [11-13]. The level of YAP was increased in NSCLC tissues, which was mainly accumulated in the nucleus with a lesser cytoplasm presence. Its overexpression in lung cancer cells promoted cell proliferation and invasion while its depletion impaired cell viability [14].

Some study reported that YAP suppressed the form of P53-Aspp1-Lats2 complex to inhibit cell apoptosis [15]. However, YAP was also reported to be a tumor suppressor as its gene locus was deleted in some breast cancers with a correlated loss of YAP protein expression [16]. In addition, YAP as a transcriptional coactivator promoting anti-tumoral effects on the basis of interacting with P73, P65 and P53-binding-2 but P53 [17]. It also identified that YAP bind P73 to regulate the protein level of BAX, meanwhile, YAP bind Itch E3 instead of P73 to inhibit its degradation [18-20]. In this study, we found that YAP expression promotes cell apoptosis in non-small cell lung cancer of P53 deletion; apoptosis is inhibited when its expression is decreased, and the expression of YAP regulates the expression of P73 and BAX which play a important role in regulating cell apoptosis, so we hypothesize YAP as a transcriptional coactivator regulates the transcriptional activity of BAX which is the downstream of P73 through binding the transcription factor P73. On the contrary, in non-small cell lung cancer of wild type P53, the effect is opposite, overexpression of YAP inhibits cell apoptosis and the expression of BAX decreases, which is may related that YAP can reduced the transcriptional activity of BAX through P53.

In conclusion, YAP as a candidate oncoprotein overexpressed in NSCLC which is important for the cell apoptosis. Additional work is needed to elucidate the therapeutic potential of multi-targeted strategies involving YAP in NSCLC.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xueshan Qiu, Department of Pathology, The First Affiliated Hospital and College of Basic Medical Sciences of China Medical University, Shenyang 110001, PR China. E-mail: xsqiu@mail.cmu.edu.cn

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