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p63 protects chondrosarcoma malignancies mainly by enhancing the expression of PTEN

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Abnormal expression of p63 has been well identified in multiple malignancies. However, little study has been done on the association between p63 and chondrosarcoma. In the current study, we mainly explored the expression of p63 in different grades of chondrosarcoma. Our data showed that p63 was significantly decreased in grade II and III chondrosarcoma compared with that of grade I chondrosarcoma and normal control. As the characteristic of grade II and III chondrosarcoma is metastasis, we then searched the function of p63 chondrosarcoma. *In vitro* study showed that overexpression of p63 significantly suppressed chondrosarcoma cell viability, migration and invasion. Meanwhile, upregualtion of p63 induced chondrosarcoma cell apoptosis. Furthermore, we showed that overexpression of p63 could significantly increase the protein expression of PTEN. In contrast, silencing of PTEN markedly reduced the protein levels of Bax, and enhanced the expression of PCNA and p27, even in cells transfected with p-p63. These data showed that p63 was a tumor suppressor mainly through regulating PTEN in chondrosarcoma cells. In summary, reduction of p63 in grade II and III chondrosarcoma enhances the malignant phenotype mainly through modulating the expression of PTEN.

1. Introduction

Due to the resistance to conventional chemotherapy and radiation therapy, chondrosarcoma remains a great challenge for patients and clinicians (Leis and Fratkin 1997; Noel et al. 2003). According to histologic grading systems, chondrosarcoma is currently divided into three stages (Li et al. 2012; Nagata et al. 2013). The overall survival rate of grade I tumor is 83% with poor metastasis, while patients with grade III tumor demonstrated only 29% overall survival rate and commonly experienced metastasis (Li et al. 2012; Nagata et al. 2013). For curative treatment, surgical removal is the only option (Noel et al. 2003; Obid et al. 2015). Therefore, it is of great importance to elucidate the underlying mechanism of high-grade chondrosarcomas, thereby exploring better therapeutic strategies to improve clinical outcome.

p63 is encoded on chromosome 3q27-28 and is a p53 homolog (Baydar et al. 2011; Jo and Fletcher 2011). Previous studies have shown that p63 is necessary for the maintenance of epithelial stem cell populations (Baydar et al. 2011; Jo and Fletcher 2011; Martin et al. 2011; Mukhopadhyay and Katzenstein 2011; Srinivasan and Parwani 2011). Furthermore, abnormal expression of p63 has been well identified in multiple malignancies, including squamous cell carcinoma, basal cell carcinoma, thymic tumors, urothelial carcinoma, and the myoepithelial components of salivary gland neoplasms (Ud Din et al. 2011; Yaskiv et al. 2011). The immunohistochemical detection of p63 is suggested to have key diagnostic values for the evaluation of epithelial neoplasms. Moreover, p63 is expressed in most soft tissue tumors, including chondrosarcoma (Jo and Fletcher 2011). However, whether p63 could be used for the diagnosis of high grade chondrosarcoma has never been explored.

In the current study, we mainly explored the expression of p63 in different grades of chondrosarcoma. To explore the function of p63, an *in vitro* study was carried out. Here, we first demonstrated that the level of p63 was reduced in grade III chondrosarcoma. P63 decreased chondrosarcoma cell migration mainly by modulating the expression of ZEB2.

2. Investigations and results

2.1. Reduced expression of p63 in grade II and III chondrosarcoma

Firstly, we evaluated the expression of p63 in normal control tissues and chondrosarcoma. IHC staining demonstrated that the level of p63 was decreased in grade II and III chondrosarcoma (Fig. 1A). Furthermore, western blot analysis also validated that the expression of p63 was suppressed in grade II and III chondrosarcoma compared with grade I chondrosarcoma and normal control (Fig. 1B).

2.2. p63 suppressed chondrosarcoma cell viability

We then explored the effects of p63 on chondrosarcoma cell viability. As shown in Fig. 2A, transfection of p-p63 increased the protein level of p63 in chondrosarcoma cells. And CCK-8 kit assay showed that overexpression of p63 significantly suppressed cell viability in 24, 48, 72 h (Fig. 2B). In contrast, transfection of siRNA targeting p63 decreased the protein level of p63 (Fig. 2C). And cell viability was markedly increased when p63 was silenced in 24, 48, 72 hours (Fig. 2D).

2.3. p63 inhibited chondrosarcoma cell migration, invasion and induced cell apoptosis

Furthermore, we found that overexpression of p63 significantly suppressed chondrosarcoma cell migration at 24, 48, 72 h (Fig. 3A). Meanwhile, cell invasion capacity was also decreased at 24, 48, 72 h after transfection of p-p63 (Fig. 3B). Annexin V-PI staining also indicated that upregulation of p63 significantly induced chondrosarcoma cell apoptosis at 24, 48, 72 h (Fig. 3C).

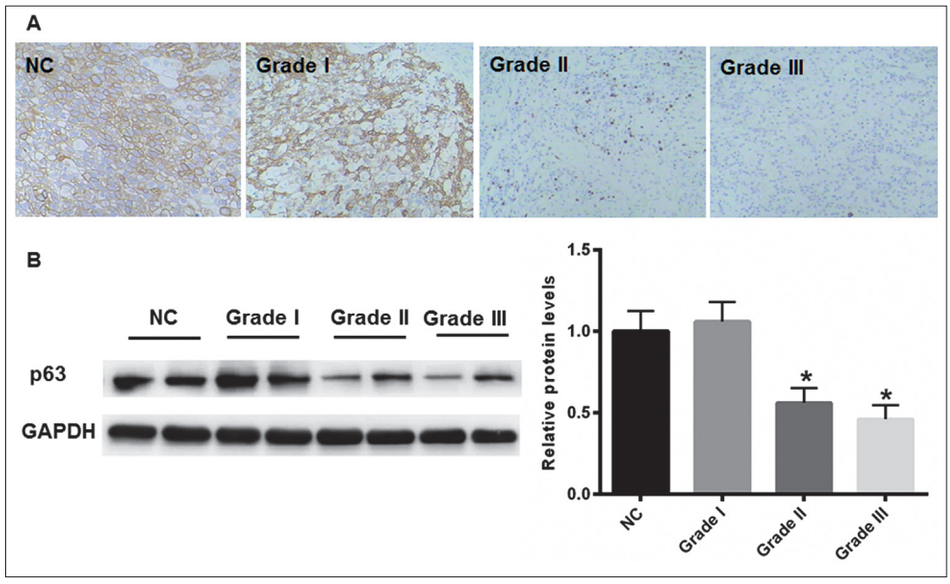

Fig. 1: Reduced expression of p63 in grade II and III chondrosarcoma. (A) IHC staining demonstrated that the level of p63 was decreased in grade II and III chondrosarcoma. (B) Western blot analysis also validated that the expression of p63 was suppressed in grade II and III chondrosarcoma. *p<0.05, **p<0.01 vs. control.

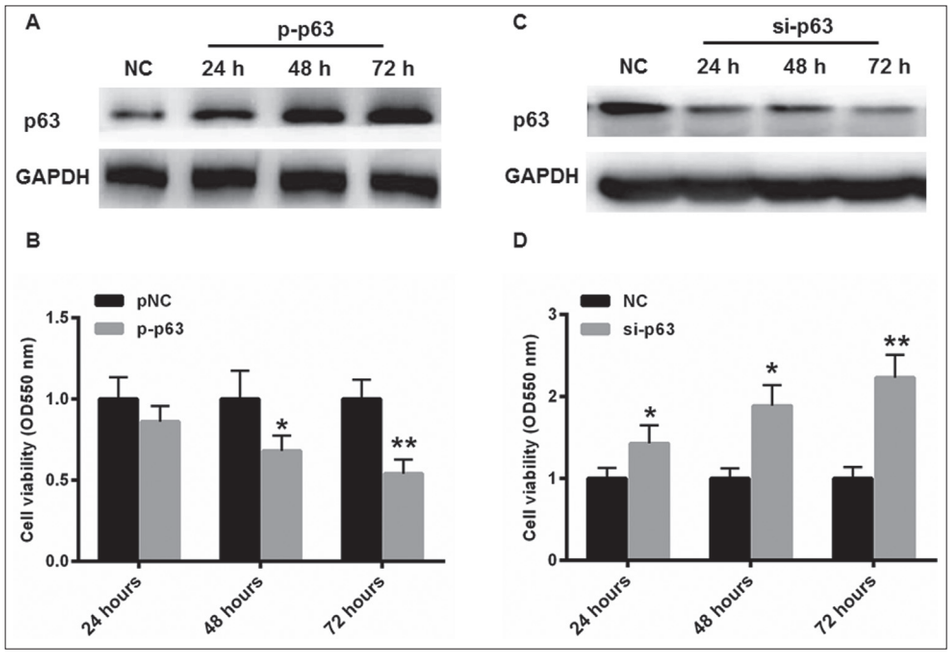

Fig. 2: p63 suppressed chondrosarcoma cell viability. (A) Transfection of p-p63 increased the protein level of p63 in chondrosarcoma cells. (B) CCK-8 kit assay showed that overexpression of p63 significantly suppressed cell viability in 24, 48, 72 hours. (C) Transfection of siRNA targeting p63 decreased the protein level of p63. (D) Cell viability was markedly increased when p63 was silenced in 24, 48, 72 hours. *p<0.05, **p<0.01 vs. control.

2.4. p63 suppressed chondrosarcoma cell malignancies through regulating PTEN

Then, we tried to explore the underlying mechanism in which p63 controls the malignancies of chondrosarcoma. Western blot analysis showed that overexpression of p63 markedly increased the protein expression of PTEN (Fig. 4A). To further explore whether p63 exerts its function through modulating the expression of PTEN, a specific siRNA targeting PTEN was selected. As shown in Fig. 4B, silencing of PTEN markedly reduced the protein levels of Bax, and enhanced the expression of PCNA and p27, even in cells transfected with p-p63.

3. Discussion

Chondrosarcoma still provides a great challenge for all disciplines of oncology (Noel et al. 2003; Rombi et al. 2013; Obid et al. 2015). At present, the histologic grading systems play a key role in the prediction of clinical behavior (Sule et al. 2015). Therefore, it is difficult for the pathologist to distinguish a benign cartilaginous lesion from a malignant one (Dallas et al. 2012; Davies et al. 2014). At present, how to provide effective advances in local or systemic adjuvant treatment is of great importance for the radiation therapist and the medical oncologist (Goda et al. 2011; Hamdi et al. 2015). Multiple studies have shown that enhanced cell proliferation rate are correlated with poor prognosis in patients with chondrosarcoma (Nikoghosyan et al. 2010; Rombi et al. 2013; Sahgal et al. 2015). Thus, identifying a novel marker of the overall proliferative activity is required in a population of neoplastic cells.

p63 is located on chromosome 3q27-29, which has three indispensable domains for their functions, including transactivation (TA) domain, DNA binding domain and oligomerization domain (Yao and Chen 2012). It is suggested that the p63 protein exerts a wide role in many physiological processes, such as transformation of induced pluripotent stem cells, oocyte death, non-small-cell lung carcinoma, autophagic cell death, epidermal morphogenesis (Bir, Aksoy Altinboga et al. 2014, Choi, Kim et al. 2014, Leao, Gomes et al. 2015, Vandormael-Pournin, Guigon et al. 2015). For instance, RUNX1 acts as an important transcription factor in human

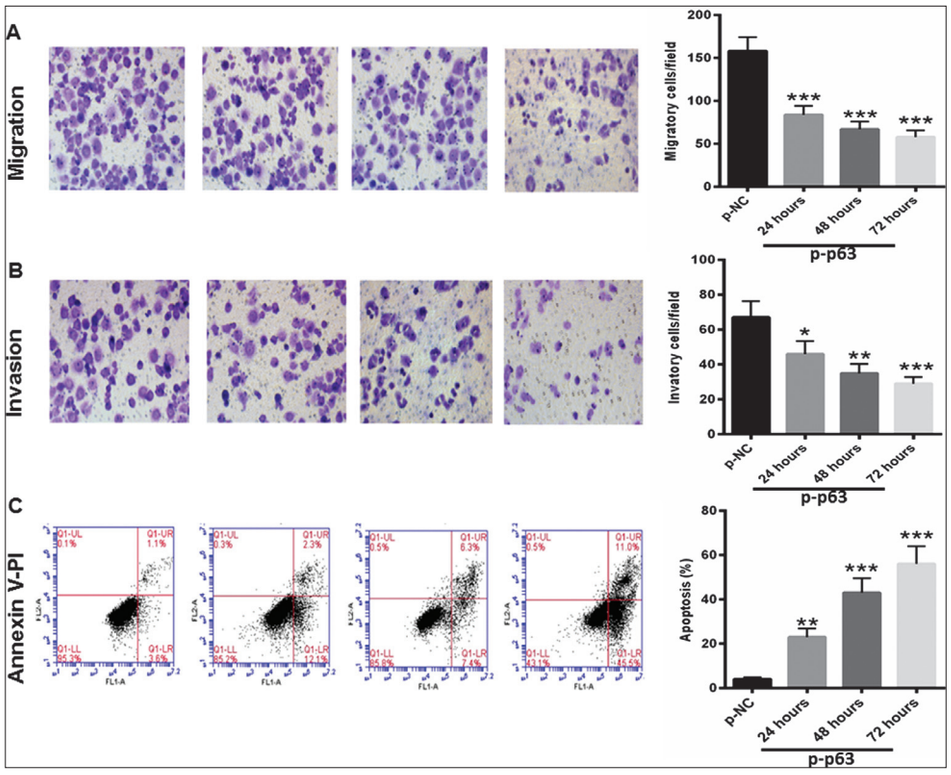

Fig. 3: p63 inhibited chondrosarcoma cell migration, invasion and induced cell apoptosis. (A) Cell migration assay. (B) Cell invasion assay. (C) Cell apoptosis assay. *p<0.05, **p<0.01 vs. control.

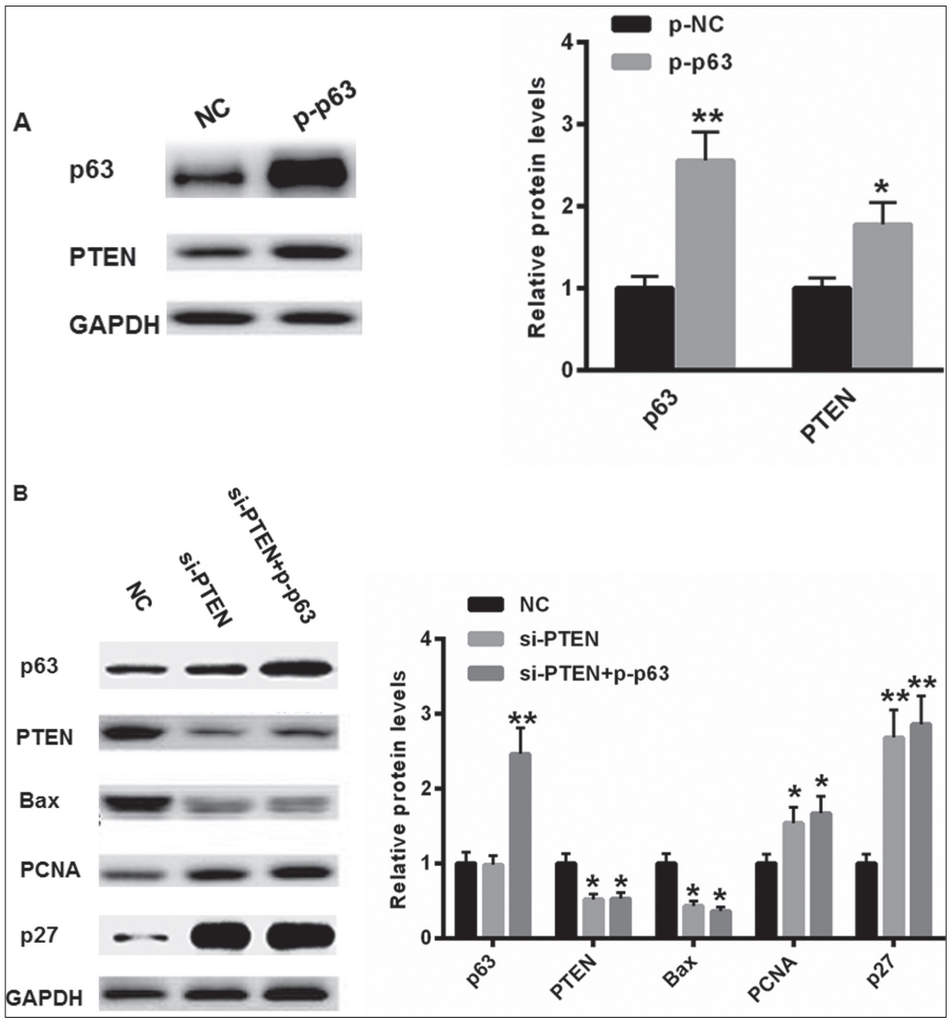

Fig. 4: p63 suppressed chondrosarcoma cell malignancies through regulating PTEN. (A) Western blot analysis showed that overexpression of p63 markedly increased the protein expression of PTEN. (B) Silencing of PTEN markedly reduced the protein levels of Bax, and enhanced the expression of PCNA and p27, even in cells transfected with p-p63. *p<0.05, **p<0.01 vs. control.

keratinocytes, thereby regulating the transition from proliferation to differentiation (Masse, Barbollat-Boutrand et al. 2012). In addition, it is also suggested that p63 and p73 co-regulated p53-dependent neural precursor cell apoptosis versus senescence, which then determines the appropriate adult neurogenesis (Fatt, Cancino et al. 2014). And studies have also demonstrated loss of p63 is associated with the more aggressive and metastatic tumors (Urist, Di Como et al. 2002, Koga, Kawakami et al. 2003). Researches elucidate that p63 is a crucial metastasis suppressor thorugh its interaction with p53 family members, thereby contributing to the tumor phenotype finally (Adorno, Cordenonsi et al. 2009, Muller, Caswell et al. 2009, Su, Chakravarti et al. 2010). However, despite its wide expression of p63 in various tissues, little study has been done on association between p63 and chondrosarcoma.

In the present study, we first explored the expression of p63 in chondrosarcoma. Our data showed that p63 was significantly decreased in grade II and III chondrosarcoma compared with that of grade I chondrosarcoma and normal control. As the characteristic of grade II and III chondrosarcoma is metastasis, we then searched the function of p63 in chondrosarcoma. An *in vitro* study showed that overexpression of p63 significantly suppressed chondrosarcoma cell viability, migration and invasion. Meanwhile, upregualtion of p63 induced chondrosarcoma cell apoptosis. These data indicated a tumor suppressor role of p63 in chondrosarcoma cells. Then, we explored the underlying mechanism by which p63 is modulating cancer cell malignancies. A previous study has shown that p63 could enhance the expression PTEN in murine liver cancer cells (Fang et al. 2016). Here, we first showed that overexpression of p63 could significantly increase the protein expression of PTEN. In contrast, silencing of PTEN markedly reduced the protein levels of Bax, and enhanced the expression of PCNA and p27, even in cells transfected with p-p63. These data showed that p63 was a tumor suppressor mainly through regulating PTEN in chondrosarcoma cells.

To conclude, reduction of p63 in grade II and III chondrosarcoma is mainly mediated through modulating the expression of PTEN.

4. Experimental

4.1. Patients

Conventional central chondrosarcomas were selected based on accepted clinicpathological and radiological criteria (Rychly et al. 2008). Peripheral-, juxtacortical-, mesenchymal-, dedifferentiated- and clear-cell chondrosarcomas were excluded. In total, specimens from 30 patients were studied, including 10 grade I patients, 10 grade II patients, 10 grade III patents. All tissue samples were confirmed by pathological analysis. The samples used in this study were approved by the ethics committee of Wuhan General Hospital of People ‘s Liberation Army.

4.2. Cell lines

Human chondrosarcoma cell line OUMS27 (purchased from ATCC) were used in the study. The cells were cultured in monolayer in RPMI 1640 (Life Technologies Invitrogen, Thermo Scientific, Waltham, MA, USA) supplemented with 10% Fetal Calf Serum and 1% Penicillin/Streptomycin at 37 °C in a humid atmosphere with 5% CO2.

4.3. Construction of vectors

The vectors overexpressing (Plasmid #27008) were purchased form Addgene (http://www.addgene.org/). The specific siRNA targeting p63 were purchased from Santa Cruz (sc-95758).

4.4. Transient transfection

Cells were seeded at 10^6 cells/well in the 6-well plates. Meanwhile, the siRNA or a non-specific siRNA (NC) were mixed with HiperFect transfection reagent (QIAGEN) and incubated at room temperature for 10 min. Then, the complex was added in to the culture medium for 48 h.

4.5. Cell proliferation assay

MG-63 cells were seeded in a 96-well plate at density of 5×10^3 cells per well. Then, the cells were transfected with pp63 or shp63 for 24, 48, 72 h, respectively. Cell viability was determined with the CCK-8 assay according to the instructions (CCK-8, Beyotime Inst Biotech, China). The cells were incubated ~4 h with CCK-8 before absorbance read for the CCK-8 proliferation assay. Each well absorbance was tested at 450 nm by microplate reader. The proliferation rate was defined in terms of the percentage of each group surviving cells compared with the untreated group.

4.6. Transwell migration and invasion assays

The cell migration assay was performed using Boyden chambers with 8-μm-pore filters (Corning, New York, USA). For the cell invasion assay, the upper chamber was pre-coated with Matrigel (BD, USA). MG-63 cells (5×10^3) treated with p-p63 or blank vector in RMPI 1640 medium for 24, 48, 72 h, respectively, were plated on the upper chamber. A volume of 600 μL medium with 20% FBS as a chemoattractant was plated in the lower chamber of the 24-well plates. Cells were then incubated under standard culture conditions for 48 h. Non-migrating and non-invading cells in the upper chamber were completely removed using a cotton swab. Cells that migrated or invaded to the lower surface of the membrane were fixed in methanol for 30 min at 37 °C and stained with 0.5% crystal violet for 1 h. Cells were quantified by counting the number of stained nuclei in five random fields by fluorescence microscopy, in triplicate.

4.7. Flow cytometry evaluation of apoptosis

Apoptosis of cells was evaluated using a BD Annexin V-FITC/PI apoptosis detection kit. After 48 h of treatment with different concentrations of nigericin, MG-63 cells were harvested by trypsinization without EDTA and washed 3 times with ice-cold PBS. Thereafter, cells were suspended in the Annexin V-binding buffer to a final concentration of 10^4 cells/ml. Cells were then incubated with 5 μL of Annexin V-FITC for 15min, followed by staining with 5 μL of a PI solution. All samples were immediately analysed by flow cytometry. Experiments were performed in triplicate.

4.8. Immunohistochemistry

Immunohistochemistry was performed according to the standard manufacturer’s protocol to measure p63 (CST) expression in normal ovarian tissues and chondrosarcoma.

4.9. Western blotting

Total cell proteins were extracted in RIPA lysis buffer (Biyuntian, Jiangsu, China) with freshly-added proteinase inhibitor cocktail and phosphatase inhibitor (Sigma, St. Louis, MO, USA) on ice for 15 min and centrifuged at 12,000 rpm for 20 min. Bicinchoninic acid (BCA, Sigma, St. Louis, MO, USA) was used to determine the concentration of total protein of EOC cells. For western blot analysis, lysates (20 μg/well) were subjected to 10% SDS-PAGE, and then fractionated proteins were transferred to PVDF membrane (Millipore, USA). The membrane was blocked in 5% non-fat skim milk for 1 h at room temperature and then incubated with primary antibodies overnight at 4 °C. The next day, samples were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Positive immunoreactive proteins were detected using an ECL kit (Thermo Fisher, USA) to visualize signals and bands. The band intensity was analysed using Quantity One analysis software (Bio-Rad, California).

4.10. Statistical analysis

Data were presented as mean±SD from three independent experiments or five mice. Statistical analysis was carried out with Student’s t test. $P < 0.05$ was considered as statistically significant difference.

Conflicts of interest: None declared.

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