p63 inhibits CD44+/CD24- cell proliferation and chemoresistance in papillary thyroid carcinoma cells

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Abstract. Thyroid cancer typically has a good prognosis; however, the risks of recurrence and chemoresistance associated with thyroid cancer remain a cause for concern. Papillary thyroid carcinoma (PTC) comprises 80% of all cases of thyroid carcinoma. A previous study reported that cluster of differentiation (CD) 44+/CD24- PTC cells may contribute to PTC recurrence and chemoresistance; however, the underlying molecular mechanisms remain elusive. In the present study, CD44+/CD24- cells were isolated from the TPC-1 PTC cell line and biological function assays revealed that CD44+/CD24- cells were significantly more proliferative and chemoresistant compared with CD44-/CD24- cells. Furthermore, the expression level of p63 was demonstrated to be negatively correlated with the expression of CD44 in PTC cells. The role of p63 in CD44+/CD24- cell proliferation and chemoresistance was investigated and, the ectopic expression of p63 was observed to significantly inhibit CD44+/CD24- cell proliferation and chemoresistance *in vitro* and *in vivo*. In conclusion, the present study indicated that CD44+/CD24- cells contribute to PTC proliferation and chemoresistance and that the suppression of p63 in CD44+/CD24- cells contributes to these effects.

Introduction

Thyroid carcinoma is the cancer with the third fastest increase in diagnosis in the US, and papillary thyroid carcinoma (PTC) comprises 80% of thyroid carcinoma (1,2). PTC occurs more often in women, and although it can occur at any age, even in childhood, the peak incidence is in patients between 30 and 50 years of age (3). PTC typically has an indolent clinical course and may be cured by thyroidectomy and radioactive iodine therapy, even if metastatic (4). The majority of patients with PTC have a good prognosis; however, a subset of patients may undergo dedifferentiation and develop a more progressive disease that consequently has a dismal prognosis (5).

The human cluster of differentiation (CD)44 gene encodes type 1 transmembrane glycoproteins involved in cell-cell and cell-matrix interactions (6). The structural heterogeneity of the gene products is caused primarily by alternative splicing of at least 10 out of 20 exons (6). Certain CD44 variant isoforms, in particular those containing CD44 variant domain 6 (CD44v6) have been implicated in tumorigenesis, tumor cell invasion and metastasis (6). A previous study reported that CD44v6 is an important indicator in PTC cell differentiation, and the expression of CD44v6 is significantly higher in malignant lesions compared with non-malignant lesions (7). CD44+/CD24- cells are more aggressive than CD44- cells, not only in PTC, but also breast and ovarian cancer (8-10). However, the underlying mechanism that promotes CD44+ cells to exhibit a more malignant phenotype remains unknown.

In the present study, the expression of a p53 family member transcription factor, p63, was demonstrated to be negatively correlated with the expression of CD44. p63 is a well-known tumor suppressor (11). In addition, the present study indicated that CD44 inhibited the activity of p63 and increased the proliferation and chemoresistance capacity of PTC cells.

Materials and methods

Ethics statement. All animal experiments were approved by the Ethics Committee of Fudan University Shanghai Medical College (Shanghai, China) and followed the National Institutes of Health guidelines on the care and use of animals.

Cell culture. TPC-1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained under standard culture conditions (37°C, 5% CO_2) in the Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS).

Western blotting. TPC-1 cells were lysed in western blot/immunoprecipitation lysis buffer (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) and all procedures were conducted according to the manufacturer's protocol. Subsequently, the cell lysates were boiled in 5X SDS-PAGE loading buffer for 10 min and then 20 µg of protein was

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resolved by 8% SDS-PAGE, transferred to a nitrocellulose membrane and blocked with 5% (m/v) bovine serum albumin (BSA; Shanghai Sangon Biological Engineering, Shanghai, China) for 1 h. The membrane was subsequently incubated with primary antibodies at 4˚C for 12 h. The following antibodies were used: CD44 (#3570, 1:1,000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA), CD24 (ab64064, 1:1,000 dilution; Abcam, Cambridge, UK), p63 (#13,109 1:1,000 dilution; Cell Signaling Technology, Inc.) and GAPDH (1:5,000 dilution; ProteinTech Group, Inc., Chicago, IL, USA). Specific secondary antibodies mouse IgG (#7076, 1:2,000 dilution) and rabbit IgG (#7074, 1:2,000) (both from Cell Signaling Technology, Inc.) were used to probe the membrane at 37˚C for 1 h. Bound antibodies were subsequently visualized with an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions using a Bio-Rad ChemiDoc XRS+ system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Stable cell construction. The vector pcDNA3.1 (+) inserted with the ORF of p63 and empty vector were purchased from Fugene (Promega Corporation, Madison, WI, USA). These plasmids were transfected into TPC-1 cells with Fugene 9 (Promega Corporation) transfection system. After 48 h transfection, these cells were treated with 1 mg/ml G418 (Shanghai Sangon Biological Engineering) for 2 weeks. These selected cells were considered stable cell lines.

Cell isolation. TPC-1 cells were washed once with PBS and then harvested with 0.05% trypsin/0.025% EDTA (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Detached cells were washed with PBS containing 1% FBS (wash buffer) and then resuspended in the wash buffer (10^6 cells/100 μl). Cells were incubated with 5 μl of Fc Blocker (#422301; BioLegend, Inc., San Diego, CA, USA) at 4˚C for 20 min. Combinations of fluorochrome-conjugated monoclonal antibodies were purchased from BD Biosciences (San Jose, CA, USA) against human CD44 (fluorescein isothiocyanate conjugated; cat no. 555478) and CD24 (phycoerythrin conjugated; cat no. 555428) or their respective isotype controls were added to the cell suspension at 1:100 dilution and incubated at 4˚C in the dark for 30 to 40 min. CD44⁺/CD24⁻ cells were sorted by flow cytometry on a FACSVantage cytometer (BD Biosciences) and analyzed using FlowJo software (version 10.1; FlowJo LLC, Ashland, OR, USA). The purity of sorted cells in this study was consistently more than 99%.

Cell Counting Kit-8 (CCK-8) cell viability assays. TPC-1 cells were seeded into a 96-well plate at $3x10^3$ cells/well with 100 μl DMEM containing 0, 2, 4, 6 and 8 μg/ml concentrations of 5-fluorouracil (5-FU; #F6627; Sigma-Aldrich, Merck KGaA) and cultured at 37˚C in an atmosphere containing 5% CO_2 for 24 h. The cell viability was quantified by the addition of 10 μl CCK-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Following 1.5 h of incubation at 37˚C in an atmosphere containing 5% CO_2 in the dark for 30 to 40 min, the plates were monitored using a Power Wave XS microplate reader (Omega Bio-Tek, Inc., Norcross, GA, USA) at an absorbance 450 nm.

Colony formation assays. TPC-1 cells (500/well) were seeded in 6-well plates and cultured in DMEM for 10 days at 37˚C in an atmosphere containing 5% CO_2. After 10 days, colonies were fixed and stained with 0.5% crystal violet (Beyotime Institute of Biotechnology) and the number of colonies was counted. Only those cell clusters containing more than 50 cells under a microscope were considered as colonies. The assay was performed in triplicate.

In vivo tumor formation assay. In this assay, 10 BALB/c (nu/nu) 4-week-old male mice were from Shanghai Laboratory Animal Center (Shanghai, China) and housed in a specific pathogen-free facility. Mice were housed with a 14/10 h light/dark cycle and provided with food and water *ad libitum*. The mice were group-housed (3-5 per cage) and maintained at a temperature of 24±1˚C and a humidity of 50±10%. A total of $1x10^6$ p63-overexpressing (p63 group) or empty vector (EV) CD44⁺/CD24⁻ TPC-1 cells (control group) were each subcutaneously injected into the right flank of mice (n=5/group, weight 17.6±0.4 g vs. 18.2±0.6 g). Tumor sizes were measured and calculated with the formula V = (length x width²)/2 once a week and mice were sacrificed for the analysis of tumor burden after 4 weeks.

Immunohistochemical staining. The resected mice tumors were fixed in methanol and embedded in paraffin and then tissues sectioned to 4 μm slides. These slides were deparaffinized gradually using 50% xylene (Meryer, Shanghai, China) and rehydrated. The sections were then incubated with 0.3% hydrogen peroxide (Jianglaibio, Shanghai, China) for 30 min at 37˚C to inhibit endogenous peroxidase activity and blocked with 10% BSA for 10 min 37˚C. Sections were incubated with an antibody targeting p63 (#13109, 1:200 dilution) or Ki-67 (#9449, 1:200 dilution) (both from Cell Signaling Technology, Inc.) at 4˚C overnight and subsequently reacted with horseradish peroxidase-conjugated secondary antibody (#13109; 1:50; Beyotime Institute of Biotechnology) for 20 min at 37˚C. Sections were stained with diaminobenzidine for 2 min at 37˚C, counterstained with hematoxylin for 30 sec at 37˚C and mounted with neutral balsam. A light microscope was used to capture representative images (magnification, x200) of the tumor tissues.

Statistics analysis. Data are expressed as the mean ± standard deviation as indicated. Student's t-test was used for comparisons between groups and P<0.05 was considered to indicate a statistically significant difference.

Results

CD44⁺/CD24⁻ PTC cells exhibit increased proliferative and chemoresistant capacity. It was previously reported that CD44⁺/CD24⁻ PTC cells obtain a more progressive phenotype compared with CD44⁻/CD24⁻ cells in PTC progression (7). In the present study, CD44⁺/CD24⁻ cells were isolated from the TPC-1 PTC cell line, and the isolated cells were validated by western blotting (Fig. 1A). Subsequently, the cell proliferation and chemoresistance capacities of the isolated CD44⁺/CD24⁻ cells were explored in comparison with those of CD44⁻/CD24⁻ cells. As shown in Fig. 1B-D, compared with CD44⁻/CD24⁻ cells, CD44⁺/CD24⁻ cells exhibited significantly

increased proliferative activity (Fig. 1B) and chemoresistance to 5-FU at specific doses (Fig. 1C and D). These data indicate that CD44+/CD24- PTC cells have a greater proliferative and chemoresistance capacity compared with CD44-/CD24- cells.

Expression of p63 is negatively correlated with the expression of CD44. p63 is a member of the p53 family of transcription factors and previous studies have revealed the suppressive roles of p63 in the field of tumor biology (12,13). The protein expression levels of p63 in TPC-1 isolated cell groups were investigated, as shown in Fig. 2A. CD44+/CD24- TPC-1 cells exhibited lower expression levels of p63 compared with CD44-/CD24- PTC cells. This finding suggested that CD44 may inhibit the activity of p63. Subsequently, the effects of overexpression of p63 in these isolated CD44+/CD24- PTC cell lines were investigated; the overexpression was demonstrated by western blotting (Fig. 2B). Cellular clone formation assays and CCK-8 cell viability assays revealed that overexpression of p63 significantly attenuated the capacities of clone formation (Fig. 2C) and chemoresistance to 5-FU at specific doses (Fig. 2D) in CD44+/CD24- TPC-1 cells compared with that in the EV group. These data suggest that CD44+/CD24- cells inhibit the activity of p63 to be more progressive than CD44-/CD24- cells.

Ectopic expression of p63 in CD44+/CD24- PTC cells inhibits xenograft tumor formation. To further investigate the inhibitory effects of p63, CD44+/CD24- p63-overexpressing TPC-1 cells and EV control TPC-1 cells were used to perform a xenograft subcutaneous tumor formation assays. As shown in Fig. 3A-C, when tumors derived from CD44+/CD24- TPC-1 cells were analyzed, those overexpressing p63 exhibited reduced tumor growth *in vivo* compared with those in the control group. There was a significant difference in tumor volume between the groups on days 21 and 28 (Fig. 3B) and tumor weight on day 28 (Fig. 3C). Furthermore, immunohistochemical staining indicated that the expression of Ki-67, a cell proliferative marker (14), was markedly lower in the p63-overexpressing group compared with the control group (Fig. 3D). These results suggest that CD44+/CD24- cells may inhibit the activity of p63, which results in increased cell proliferation.

Discussion

In the present study, CD44+/CD24- PTC cells were indicated to have a lower activity of p63 compared with CD44-/CD24- PTC cells, which increased the aggressive malignant phenotype of these cells, particularly regarding proliferation and chemoresistance. CD44 s are a polymorphic family of cell surface glycoproteins that are implicated in cell-to-cell and cell-to-matrix adhesion interactions (15). CD44 has been reported to be involved in tumor metastasis and invasion in breast cancer, the regulation of epithelial-mesenchymal transition and modulation of phosphoinositide 3-kinase/Akt/mechanistic target of rapamycin and activation of Wnt canonical signaling (15-17). In the present study, CD44+/CD24- cells were isolated from the PTC cell line TPC-1. The present study findings, which indicated that CD44+/CD24- cells are more proliferative and chemoresistant to 5-FU than are CD44-/CD24- cells, are consistent with
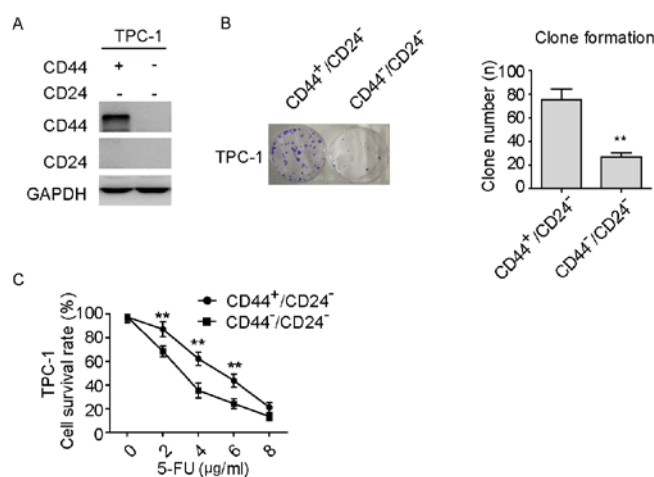

Figure 1. CD44+/CD24- PTC cells are progressive. (A) Western blotting identified the isolated CD44+/CD24- and CD44-/CD24- TPC-1 cells. (B) Colony formation capacities of the isolated types of TPC-1 cells were analyzed using soft agar cellular colony formation assays. Data are shown as mean ± standard deviation (n=3). (C) The chemoresistance of TPC-1 cells to 5-FU were analyzed by Cell Counting Kit-8 cell viability assays. Data are shown as mean ± standard deviation (n=3). **P<0.01 vs. control. CD, cluster of differentiation; 5-FU, 5-fluorouracil; PTC, parathyroid carcinoma.
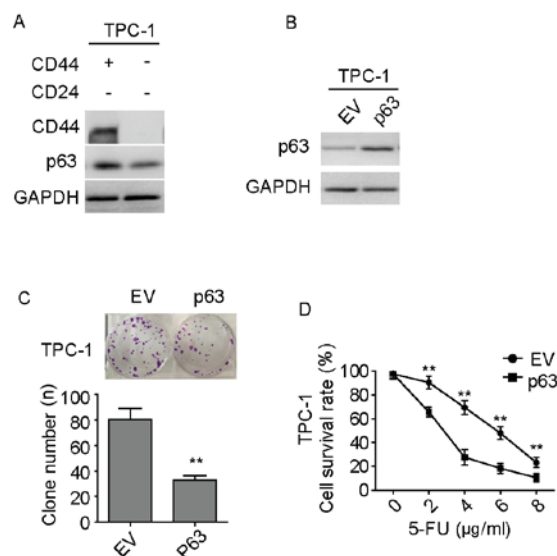

Figure 2. p63 is negatively correlated with the expression of CD44. (A) Protein expression levels of p63 were markedly higher in isolated CD44-/CD24- TPC-1 PTC cells compared with CD44+/CD24- cells. (B) Protein expression levels of p63 were markedly increased in p63-overexpressing TPC-1 CD44-/CD24- cells. (C) Ectopic expression of p63 causes a significant reduction in the colony formation capacity of the TPC-1 cell line. (D) The chemoresistance of p63-overexpressing TPC-1 cells to 5-FU were analyzed by Cell Counting Kit-8 cell viability assays. Data are expressed as the means ± standard deviation (n=3). **P<0.01 vs. control. CD, cluster of differentiation; 5-FU, 5-fluorouracil; PTC, parathyroid carcinoma.

previous studies (15,16). These findings suggest that CD44 may be a pivotal oncoprotein in patients with malignant PTC.

To the best of our knowledge, the mechanism by which CD44 exerts an aggressive role in malignant PTC progression has not been reported to date. In the present study, the CD44+/CD24- cells exhibited significantly reduced protein expression levels of p63 compared with CD44-/CD24- cells. This finding indicated that CD44+/CD24- cells may have an attenuated expression
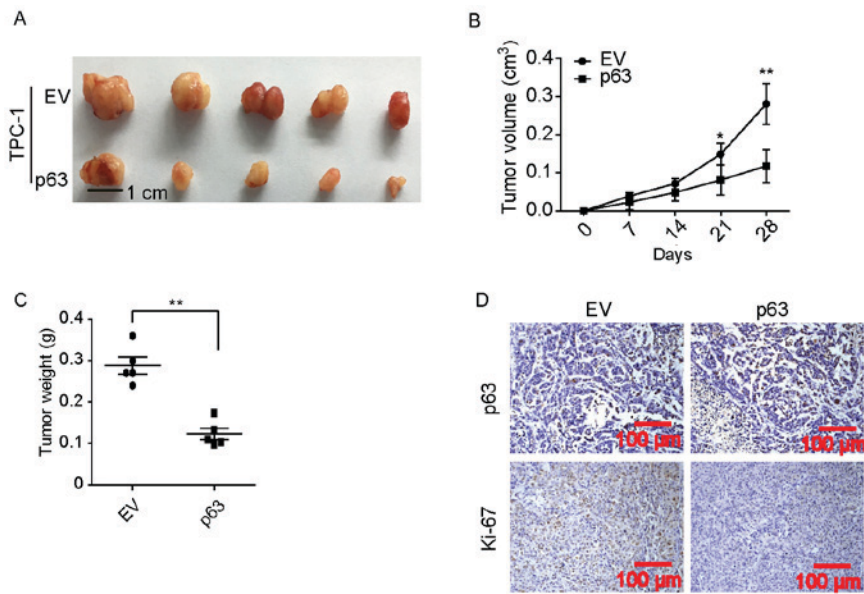
Figure 3. Overexpression of p63 attenuates the tumorigenic capacity of CD44$^+$/CD24$^-$ TPC-1 PTC cells. (A) p63 overexpression attenuated xenograft tumor growth in CD44$^+$/CD24$^-$ PTC cells. (B) Tumor volume and (C) tumor weight at day 28 from mice injected with p63-overexpressing CD44$^+$/CD24$^-$ and control empty vector (EV) CD44$^+$/CD24$^-$ PTC cells. n=5/group. (D) Immunohistochemical staining of p63 and Ki-67 in indicated subcutaneous tumors. Magnification, x200. Data are expressed as mean ± standard deviation. *$P<0.05$ vs. control. **$P<0.01$ vs. control. CD, cluster of differentiation; PTC, parathyroid carcinoma.

of p63, which increased the aggressiveness of the cells. The ectopic expression of p63 in CD44$^+$/CD24$^-$ cells supported this hypothesis; overexpression of p63 reduced CD44$^+$/CD24$^-$ cell proliferation *in vitro* and *in vivo*. The results considered together indicate that malignant PTC cells highly expressed CD44, and exhibited aggressive malignant phenotypes, which were likely associated with the inhibition of p63.

In conclusion, the present study indicates that CD44$^+$/CD24$^-$ cells primarily contribute to PTC proliferation and chemoresistance. Moreover, the results suggested that p63 was suppressed in CD44$^+$/CD24$^-$ cells and that this suppression was associated with PTC cell proliferation and chemoresistance.

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