Original Article

Knockdown of REGγ inhibits proliferation by inducing apoptosis and cell cycle arrest in prostate cancer

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Received March 28, 2017; Accepted July 11, 2017; Epub August 15, 2017; Published August 30, 2017

Abstract: Prostate cancer (PCa) is the most common malignant tumor and the second leading cause of cancer related death among men in western countries. REGγ, a proteasome activator, is reported to play important roles in various human cancers. However, the expression patterns and potential roles of REGγ in prostate cancer have never been reported. In this study, we found for the first time that REGγ is overexpressed in prostate cancer tissues and cell lines at both protein and mRNA levels. In addition, we demonstrated that knockdown of REGγ significantly inhibited cell proliferation and induced apoptosis and cell cycle arrest in PCa cell lines PC-3 and DU145. Moreover, we observed that the expressions of P21 were increased while the levels of cyclinD1 and bcl-2 were decreased after knockdown of REGγ in PCa cells. And the stabilization of P21 was enhanced after REGγ knockdown in PC-3 cells. In summary, our findings suggest that REGγ may play important roles in prostate cancer and may serve as a novel therapeutic target in the treatment of PCa patients.

Keywords: Prostate cancer, REGγ, proliferation, apoptosis, cell cycle

Introduction

Prostate cancer (PCa) is the most common malignant tumor and the second leading cause of cancer related death among men in western countries [1]. It is reported that the 5-year survival rate of early stage prostate cancer is over 99% while that of advanced metastatic disease is only 28% [2]. Nowadays, several therapy methods have been applied to advanced PCa treatment, such as androgen deprivation therapy [3] and docetaxel chemotherapy [4]. However, the prognosis of advanced PCa patients is still poor. Thus, the identification of novel molecule involved in the tumorigenesis and development of prostate cancer will be helpful for early diagnosis and treatment of PCa patients.

REGγ, also known as PA28γ, PSME3 and Ki antigen, is a member of the 11 s family of proteasome activators that bind to and activate 20 s core proteasome [5, 6]. It was shown to regulate the degradation of a series of important proteins in an ubiquitin- and ATP-independent manner, including steroid receptor coactivator 3 (SRC-3) [7], the cell cycle inhibitors (p16, p21 and p53) [8, 9] and c-Myc [10]. Recently, accumulating evidences suggest the importance of REGγ in the carcinogenesis and development of various human cancers including breast cancer [11], thyroid cancer [12] and skin cancer [13]. However, the expression pattern and biological functions of REGγ in prostate cancer have never been investigated.

In the present study, we are aimed to investigate the expression and biological roles of REGγ in prostate cancer. We found that REGγ is overexpressed in prostate cancer tissues and cell lines compared with the adjacent normal tissues and the normal prostate epithelial cell line RWPE-1. In addition, we demonstrated that knockdown of REGγ significantly inhibited proliferation and induced apoptosis and cell cycle arrest in PCa cell lines PC-3 and DU-145. Furthermore, we observed that knockdown of REGγ altered multiple apoptosis and cell cycle related proteins expression in PCa cells. And the stabilization of P21 was enhanced after knockdown of REGγ in PC-3 cells. Our results
suggest that REGγ is upregulated and may act as an oncogenic protein in prostate cancer.

**Materials and methods**

**Tissue samples**

Eighteen paired prostate cancer tissues (T), adjacent tissues (AT) and the matched normal prostate tissues (N) were obtained from the Department of Urology, Shanghai Tenth People’s Hospital, Tongji University. These patients did not receive any local or systemic treatment before operation. All fresh tissue samples were fixed in 4% paraformaldehyde for IHC staining or immediately snap-frozen in liquid nitrogen and stored in liquid nitrogen until further use. Our work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Written informed consent was obtained from all patients, and this study was approved by the Ethics Committees of Shanghai Tenth People’s Hospital.

**Cell lines**

Prostate cancer cell lines (PC-3, DU145 and LNCaP) and normal prostatic epithelial cell (RWPE-1) were obtained from the American Type Culture Collection (ATCC, Rockville, USA). PC-3, DU145 and LNCaP were maintained in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco), 50 U/ml of penicillin and 50 μg/ml of streptomycin (Invitrogen, USA). RWPE-1 cells were cultured in keratinocyte serum-free media supplemented with bovine pituitary extract (0.05 mg/ml) and epidermal growth factor (5 ng/ml). All cell lines were incubated in a humidified incubator containing 5% CO₂ at 37°C.

**Immunohistochemistry (IHC)**

For IHC staining, fresh tissue samples were fixed in 4% paraformaldehyde, dehydrated through a graded series of ethanol solution and embedded in paraffin. After routine rehydration, antigen retrieval, and blocking, the sections were incubated with primary antibody against REGγ (Invitrogen, USA) at 4°C overnight. Then, the sections were incubated with biotinylated goat anti-rabbit antibody IgG for 20 min at room temperature and for 30 min with Streptavidin-HRP peroxidase. Subsequently, Diaminobenzidine (DAB)-H₂O₂ was used as a substrate for the peroxidase enzyme.

**Western blot**

Total proteins were extracted from tissue samples or cells, separated by 10% sodium lauryl sulfate-polyacrylamide gels (SDS-PAGE) and transferred onto a nitrocellulose (NC) membrane. Membranes were blocked with 5% non-fat milk in PBS for 1 h at room temperature and then incubated with primary antibody against REGγ (Invitrogen, USA), P21 (BD Biosciences, Erembodegem, Belgium), cyclinD1 and bcl-2 (Abcam, Cambridge, MA). β-actin (Abcam, Cambridge, MA) was used as an internal control. After incubated with a fluorescent-labelled secondary antibody (Jackson immuno research; 1:5,000 dilutions) for 1 h at 4°C, membranes were washed three times and the specific signals were visualized by a LI-COR Odyssey Infrared Imaging System. Three independent experiments were carried out.

**RNA isolation and qRT-PCR**

Total RNA was extracted from human tissues or cultured cells with Trizol reagent (Invitrogen, CA, USA), and the corresponding cDNA was generated with the cDNA synthesis kit (Takara, Kyoto, Japan) according to the manufacturer's instructions. Quantitative real time-PCR (qRT-PCR) was performed using SYBR Green PCR Kit (Takara Biotechnology, Dalian, China) with an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). β-actin was used as internal control for REGγ. The primer sequences (Biosune Biotech, Shanghai, China) were as follows: 5'-AAGGGTAGC-CTTCAAGGAGC-3' (sense) and 5'-AGTGGATTCT- GATGTTGTCATGG-3' (antisense) for REGγ; 5'-CTAGATGATATCCATTGCTCCAG-3' (sense) and 5'-CTCCTACTTGTGCTCTGC-3' (antisense) for β-actin. The relative expression level of REGγ mRNA was determined by 2-ΔΔCt method normalized to β-actin. Assays were repeated for three times.

**Transient transfection**

Small interfering RNA specifically targeting human REGγ (si-REGγ) and non-specific negative control oligos (si-NC) were purchased from GenePharma (Shanghai, China). Cell transfe-
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Tions were performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc. USA) according to the manufacturer’s instructions. The sequences of si-REGγ were: 5’-CAG-AAGACUUGGGCAAATT-3’ (sense) and 5’-UU-UGCCACCAAGUCUUGTGTT-3’ (antisense). The sequences of si-NC were: 5’-UUCUCGGAAACG-UGUCACGTT-3’ (sense) and 5’-ACGUAGCAC-GUUCGGAATT-3’ (antisense). Total RNA or protein was extracted after 48 h following transfection.

MTT assay

Cell proliferation was evaluated by the MTT assay following the manufacturer’s guideline. Briefly, the cells (PC-3 and DU145) were effectively transfected with si-REGγ or si-NC and seeded into 96-well plates. After incubated for different time, 100 μl of full medium containing 0.5 mg/mL MTT (Sigma-Aldrich, St. Louis, Mo, USA) were added to each well and maintained at 37°C for 4 h. Subsequently, 150 μl DMSO was used to each well to dissolve the formazan crystals and the absorbance at 490 nm was measured using a microplate reader (SpectraMax 190; Molecular Devices Sunnyvale, CA, USA). Each experiment was performed in triplicate.

Colony formation assay

For colony formation assays, cells were transfected respectively with si-NC or si-REGγ for 24 h and plated in 6-well plates at 1 × 10^5/well for 2 weeks. The plates were then washed twice with cold PBS, fixed with methanol, and stained with 0.1% crystal violet (0.1% in 20% methanol). Images of stained tumor cell colonies were recorded with a digital camera.

Apoptosis and cell cycle analysis

Cell apoptosis was measured by flow cytometry using Annexin V-FITC Apoptosis Kit (BD Biosciences, Erembodegem, Belgium) in accordance with the manufacturer’s instructions. Briefly, transfected cells were collected, washed twice with cold PBS and resuspended in Annexin V-binding buffer. After that, cells were stained with fluorescein isothiocyanate (FITC) and propidium iodide (PI) in the dark at room temperature for 15 min. Apoptosis rate were also detected by using BD FACS Calibur (Beckman Coulter, CA, USA). Cell cycle distribution was measured by propidium iodide (PI) staining and analyzed by flow cytometry. After 48 h of transfection, cells were harvested and washed twice with pre-cooled PBS. Then, cells were fixed in 70% ethanol at 4°C overnight and washed with cold PBS. Cells were collected and resuspended in PBS containing PI and 50 μg/ml RNase A (Sigma-Aldrich) in the dark at 37°C for 30 min. Cell cycle distribution was analyzed by flow cytometry using BD FACS Calibur. Three independent experiments were conducted.

Statistical analysis

Data were analyzed by SPSS software (Version 17.0, SPSS, Inc., Chicago, IL, USA) and GraphPad Prism software (Version 5.0, GraphPad Prism Software Inc., San Diego, CA). All continuous data were presented as the mean ± standard deviation (SD) and analyzed using Student’s t-test. *P<0.05 were considered statistically significant.

Results

REGγ is overexpressed in PCa tissues and cell lines at both protein and mRNA levels

To study the potential roles of REGγ in the development of prostate cancer, we detected the expression patterns of REGγ in PCa tissues and cell lines at both protein and mRNA levels. Results of Immunohistochemistry (IHC) indicated that REGγ is significantly upregulated in prostate cancer tissues compared with normal prostate tissues (Figure 1A). In addition, data from western blot and qRT-PCR showed that both the protein and mRNA levels of REGγ are higher in PCa tissues than that in normal prostate tissues (Figure 1B, 1C). Furthermore, we found that REGγ is markedly overexpressed in three PCa cell lines (PC-3, DU145 and LNCaP) compared to normal prostatic epithelial cell RWPE-1 (Figure 1D, 1E). The overexpression of REGγ in prostate cancer was also confirmed by Oncomine database (Figure 1F). These results suggest that REGγ may play an oncogenic role in prostate cancer development.

Knockdown of REGγ inhibited proliferation in PCa cells

Small interfering RNA targeting REGγ (si-REGγ) was transfected to suppress the expression of
REGγ in PCa cells, and non-specific negative
control oligos (si-NC) was used as a control. The
inhibition of REGγ expression was confirmed by
qRT-PCR and western blot (Figure 2A, 2B). MTT
and colony formation assay were performed to
investigate cell proliferation after knockdown
of REGγ. Data of MTT assay showed that knock-
down of REGγ significantly inhibited prolifera-
tion in PCa cell lines PC-3 and DU145 (Figure
2C, 2D). In addition, the colony formation rate
of cells was significantly inhibited following
REGγ knockdown (Figure 2E, 2F).
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Knockdown of REGγ induced apoptosis and cell cycle arrest in PCa cells

As the proliferation of PCa cells was inhibited after REGγ knockdown, we presumed that this procedure may correlate with cell apoptosis and/or cell cycle distribution, which were performed by flow cytometry. Results indicated that knockdown of REGγ remarkably promoted apoptosis in PCa cells (Figure 3A, 3B). Moreover, we observed that the percentage of cells was significantly increased in G0/G1 phase while decreased in G2/M phase when REGγ expression was inhibited (Figure 3C, 3D). These data suggest that knockdown of REGγ may inhibit proliferation by inducing apoptosis and cell cycle arrest in PCa cells.

Knockdown of REGγ altered multiple apoptosis and cell cycle related protein levels in PCa cells

To further understand the effect of REGγ on PCa cells proliferation, apoptosis and cell cycle

Figure 2. Knockdown of REGγ inhibited proliferation in PCa cells. (A and B) Relative mRNA levels (A) and protein expressions (B) of REGγ in PCa cells (PC-3 and DU145) after 48 h transfection of si-NC or si-REGγ. (C and D) Cell proliferation of PC-3 and DU145 after REGγ knockdown as determined by MTT assay. (E and F) Colony formation rates of PC-3 and DU145 following knockdown of REGγ. Data are represented as mean ± SD. *P < 0.05.
distribution, we analyzed several apoptosis and cell cycle related protein expressions in PCa cells after REGγ knockdown. We found that the expressions of P21 were increased while the expressions of cycinD1 and bcl-2 were decreased after transfected with si-REGγ in PCa cells (Figure 4A). Previous studies demonstrated that REGγ can regulate the stabilization of P21 via directly degradation, we verified that in PC-3 cells. After transfected with si-NC or si-REGγ, PC-3 was cultured with full media supplemented with 100 μg/ml cycloheximide (CHX, Amresco, Solon, OH) for different time period and the protein level of P21 was analyzed by western blot. We observed that knock down of REGγ enhanced the stabilization of P21 in PC-3 cells (Figure 4B), which was consistent with previous studies. These results showed that REGγ may
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**Discussion**

Although androgen deprivation therapy (ADT) is one of the major treatments for PCa, a large proportion of the cancer cases develop castration-resistance, which is characterized by increases in resistance to apoptosis and distant metastasis [14]. And no standard therapy is effective for advanced prostate cancer. Despite the advances in the research of prostate cancer, the molecular mechanism of its pathogenesis is poorly understood, and prostate-specific antigen (PSA) still remains the most effective molecular marker in the diagnosis and prognosis prediction in PCa patients [15]. Thus there is an urgent need to seek more novel biomarkers involved in the development of prostate cancer for the diagnosis and treatment of PCa patients.

Murata et al. reported that the REGγ-deficient mice showed a retarded growth compared with that of REGγ (+/-) or REGγ (+/+)) mice, suggesting its regulatory effects on cell proliferation and body growth [14]. An increasing number of studies have shown that REGγ is upregulated in multiple human cancers including pancreatic cancer [15], lung cancer [16] and hepatocellular carcinoma [17], indicating its pivotal role in cancer development. In this study, we found for the first time that REGγ is overexpressed in prostate cancer tissues and cell lines at both mRNA and protein levels by using immunohistochemical analysis, western blot analysis, qRT-PCR and Oncomine data mining. In addition, we explored the biological effects of REGγ on PCa cells proliferation, apoptosis, and cell cycle distribution. We demonstrated that knockdown of REGγ significantly inhibited the proliferation, induced apoptosis and promoted cell cycle arrest in PC-3 and DU145 cells.

These results suggest that REGγ is upregulated and may act as an oncogenic protein in prostate cancer.

To the best of our knowledge, REGγ exerts its function by degrading target proteins and regulating multiple cancer-related pathways [18]. We analyzed several apoptosis and cell cycle related proteins expression in PCa cells after REGγ knockdown. We observed that the expressions of P21 were increased while the expressions of cyclinD1 and bcl-2 were decreased after knock down of REGγ in PCa cells. Moreover, knock down of REGγ enhanced the stabilization of P21 in PC-3 cells. These suggest that REGγ may affect cell proliferation, apoptosis and cell cycle distribution by regulating the levels of P21, cyclinD1 and bcl-2 in prostate cancer. As the weakness of our study, we didn’t comprehensively disclose the mechanism by which REGγ plays its roles in prostate cancer. Thus, further study is needed to fully understand the importance of REGγ in the pathogenesis and development of prostate cancer.

In conclusion, our data showed that REGγ is overexpressed in prostate cancer tissues and cell lines, and knockdown of REGγ significantly
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inhibited proliferation and induced apoptosis and cell cycle arrest in PCa cells. In addition, REGγ may exert its functions by regulating the expression levels of P21, cyclinD1 and bcl-2 in prostate cancer. These findings suggest that REGγ may play important roles in PCa and may serve as a novel therapeutic target in the treatment of prostate cancer patients.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (C050201 and 81602216) and Natural Science Foundation of Shanghai (16ZR1426500). This study was also funded by the National Basic Research Program (2015CB910403) and Shanghai natural science foundation (12-ZR1409300).

Disclosure of conflict of interest

None.

Authors’ contribution

Conceived and designed the experiments: XDY, LL, JHZ. Performed the experiments: SJC, LSW, CX. Analyzed the data: SJC, HC, BP, YFX. Contributed reagents/materials/analysis tools: BP, YFX, XDY, JHZ. Wrote the paper: SJC, LSW, LL.

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