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Ribonucleotide Reductase Subunit p53R2 Regulates Mitochondria Homeostasis and Function in KB and PC-3 Cancer Cells

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Abstract

Ribonucleotide reductase (RR) is a rate-limiting enzyme that catalyzes *de novo* conversion of ribonucleotide 5'-diphosphates to the corresponding 2'-deoxynucleotide, essential for DNA synthesis and replication. The mutations or knockout of RR small subunit, *p53R2*, results in the depletion of mitochondrial DNA (mtDNA) in human, implying that *p53R2* might play a critical role for maintaining mitochondrial homeostasis. In this study, siRNA against *p53R2* knockdown approach is utilized to examine the impact of *p53R2* depletion on mitochondria and to derive underlying mechanism in KB and PC-3 cancer cells. Our results reveal that the *p53R2* expression not only positively correlates with mtDNA content, but also partakes in the proper mitochondria function, such as ATP synthesis, cytochrome c oxidase activity and membrane potential maintenance. Furthermore, overexpression of *p53R2* reduces intracellular ROS and protects the mitochondrial membrane potential against oxidative stress. Unexpectedly, knockdown of *p53R2* has a modest, if any, effect on mitochondrial and total cellular dNTP pools. Taken together, our study provides functional evidence that mitochondria is one of *p53R2*-targeted organelles and suggests an unexpected function of *p53R2*, which is beyond known RR function on dNTP synthesis, in mitochondrial homeostatic control.

Keywords

p53R2 (RRM2B); mitochondrial DNA (mtDNA) synthesis; ATP synthesis; membrane potential; cytochrome c oxidase

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Introduction

Ribonucleotide reductase (RR) plays an essential role in catalyzing the conversion of ribonucleoside diphosphates to the corresponding 2'-deoxyribonucleoside diphosphates, a rate-limiting step in the production of 2'-deoxyribonucleotide 5'-triphosphates (dNTPs) required for DNA synthesis and repair [1]. In humans, a large subunit (RRM1) and two small subunits (RRM2 and p53R2) of RR have been extensively characterized [2]. RRM1 contains substrate and allosteric effector binding sites, constituting the RR holoenzyme activity and substrate specificity [3–5]. p53R2 encodes a protein with striking similarity to RRM2 [2]. p53R2 reportedly participates in the DNA damage response by forming a RR holoenzyme with RRM1 and replenishing dNTPs upon the exposure to UV, γ -irradiation or adriamycin in a p53-dependent manner [6].

Genetic studies have shown that p53R2-null mice died from severe renal failure by 14 weeks of age with a greater number of apoptotic cells were observed in the kidneys at the age of 8-week-old [7]. In addition, a direct correlation between p53R2 mutations and muscle mitochondrial DNA depletion is established in children with mitochondrial depletion syndrome [8]. Collectively, these results suggest the importance of p53R2 in maintaining the integrity of mtDNA function in nonproliferating cells. However, how p53R2 regulates mitochondria homeostasis and whether this regulation is dependent on its RR activity remains unclear. Since p53R2 is regulated by p53 for DNA repair, we wonder whether the status of p53, wild-type or mutant, will affect p53R2's regulation function on mitochondrial homeostasis. We observed the depletion of p53R2 resulted in reducing mtDNA content in KB (p53 wild type) and PC-3 (p53 truncated) cancer cells. Additionally, p53R2 affects mitochondrial function, such as ATP synthesis, mitochondrial cytochrome c oxidase activity and membrane potential, and protects the mitochondrial membrane against oxidative stress.

Materials and Methods

Cell culture, siRNA knockdown assay and plasmid construction

Human oropharyngeal carcinoma KB cells (p53 wild-type) or human prostate cancer PC-3 cells (p53 mutated) were purchased from America Tissue Culture Collection. The cells were cultured in RPMI 1640, supplemented with 10% fetal bovine serum and 1% penicillin in a 5% CO₂ atmosphere at 37°C. KB or PC-3 cells (5×10^5 per well) were transfected with 60 pmol p53R2 siRNA or scrambled siRNA (Santa Cruz Biotechnology) by Lipofectamine RNAiMAX (Invitrogen) as reported previously [9] (p53R2 siRNA is a pool of 3 target-specific 19–25 nt siRNAs designed to knock down gene expression. The siRNA duplex sense strand is listed in Supplemental Table S1). At 48 h post transfection, cells were collected. The knockdown of p53R2 was measured by quantitative reverse transcription-PCR (q-RT-PCR) and Western blot analysis. KB cells were transfected with pcDNA3.1 vector (KB-vector), p53R2 sense (KB-p53R2S) or antisense (KB-p53R2AS) as described [10].

Preparation of mitochondrial extracts (ME) and western blot analysis

Preparation of cell lysates, mitochondrial and cytosolic in cultured cells was described previously [11]. Western analyses were performed as previously described [12]. The antibodies used in this study were as follows: GAPDH (Santa Cruz Biotechnology), β -ACTIN (Santa Cruz Biotechnology), COX IV (Cell Signaling), RRM1 (Invitrogen) or p53R2 (ProSci). The rabbit p53R2 antibody was raised against a human p53R2 synthetic peptide (Acetyl-PERPEAAGLDQDERSC-amide; amino acids 4–18) conjugated to keyhole limpet hemocyanin (Rockland Immunochemicals). The anti-sera were then purified by

immunoaffinity chromatography using human p53R2 peptide coupled to agarose beads (Rockland Immunochemicals).

Quantitative reverse transcriptional PCR (q-RT-PCR) and real-time quantitative PCR (Q-PCR)

The knockdown of p53R2 was measured by q-RT-PCR [9]. mtDNA content was measured by Q-PCR as described [8, 9]. The mitochondrial *COX-1* or *ND1* gene was used as reference to measure mtDNA content in cancer cells. *PDE6B* or β -*GLOBIN* gene was quantified to indicate nuclear DNA content. The primer pairs used in this study are listed in Table S2.

In vitro RR activity assay and dNTPs pool assay

RR activity and dNTP pool were assessed using a [³H] CDP reduction assay and an optimized dNTP pool assay as described previously, respectively [13, 14]. Mitochondrial extracts were then isolated and the mitochondrial dNTPs pool was measured as the whole cell dNTP pool extraction procedures.

Inner mitochondrial membrane potential analysis, free ATP determination assay and cytochrome C oxidase activity assay

Cellular inner mitochondrial membrane potential was measured using JC-1 staining (Invitrogen) as previously described [15]. The distribution of red and green fluorescence from JC-1 was displayed in a two-color contour plot and the red signals or ratio of red signals to green signals were calculated. Cellular ATP levels were measured using the Rapid bioluminescent determination ATP assay kit as described by manufactory instruction (BioAssay System) and the luminescence was measured using a luminometer (Berthold). Mitochondrial extracts were isolated from 2×10⁷ cells at 48 h post transfection of scramble or p53R2 siRNA. Mitochondrial cytochrome c oxidase activity was measured and calculated using the cytochrome c oxidase assay kit as described by manufactory instruction (Sigma).

Fluorescence assays of carboxy-H2DCFDA oxidation

Carboxy-H2DCFDA (Invitrogen) is an indicator for reactive oxygen species (ROS) that do not fluoresce until hydrolyzed by esterases and oxidation occurs within the reaction system. Ten thousand KB-vector and KB-p53R2S cells were plated in a 96-well cell culture plate and incubated at 37°C for 24 hours. Following incubation, cells were treated with H₂O₂ for another 24 hours. Cells were washed with HBSS without phenol red. Carboxy-H2DCFDA (10 μmol/L) was added and cells were further incubated for 30 minutes. The fluorescence of the oxidized form of carboxy-H2DCFDA was measured using a fMax microplate reader (Molecular Devices) with a fluorescence excitation of 485 nm and emission at 538 nm.

Results

p53R2 expression level is associated with mtDNA content in KB and PC-3 cells

Previous studies have suggested that mutation or deletion of *p53R2* gene causes severe mitochondrial DNA depletion in humans who do not have cancer. This means p53R2 is essential for maintaining mitochondrial DNA integrity in normal cells [2, 8]. To elucidate this function of p53R2 in human cancer cells, mtDNA content was measured in KB and PC-3 cancer cells with or without p53R2 knockdown. q-RT-PCR and Western blot analyses showed that p53R2 mRNA and protein level were significantly decreased at 48h after transfection with si-p53R2 (Fig. 1A, 1B). *COX-1*, a mitochondrial gene of complex IV, levels were measured to determine the effect of p53R2 knockdown on mtDNA content by Q-PCR in KB and PC-3 cells. A decrease of 58.3% and 65.1% in mtDNA content ($p < 0.05$), without affecting nuclear *PDE6B* amount, was detected in KB and PC-3 cells,

respectively, upon the downregulation of p53R2 expression (Fig. 1C). In order to further validate this finding, we did quantitative PCR analysis to measure relative gene copy number of *ND1* (mitochondrial gene) to β -*GLOBIN* (nuclear gene). Consistently, a 69.9% and 72.6% decrease in the content of *ND1* was obtained in KB and PC-3 cells, respectively (Fig. 1D). There are no differences between the mock and scrambled siRNA treated cells (Fig. 1C&1D). Our result indicates a conserved function of p53R2 in maintenance of mtDNA content in cancer cell line regardless of p53 status.

RR activity can not be detected in mitochondria and attenuation of p53R2 expression has little effect on mitochondrial dNTP pools in both KB and PC-3 cells

To explore the functional role of p53R2 in mitochondria, the RR activity of mitochondrial extracts (ME) and cytosol extracts (CE) were measured. Compared to the CE of KB and PC-3 cells, RR activity was almost undetectable in the ME of these two cells (Fig. 2A). To further elucidate why RR activity was barely detectable in mitochondria, protein extracts from the mitochondria were analyzed by Western blot. We found that RRM1 was almost nonexistent in the ME (Fig. 2B), supporting that RR activity was not detected due to a lack of RRM1/p53R2 holoenzyme formation in mitochondria. The total dNTP pool in mitochondria was only about 4 to 7% of the total pool size (Fig. 2C compare to 2D; 2E compare to 2F). To determine whether inhibition of p53R2 expression affects mitochondrial dNTP pools, the mitochondrial and whole cell dNTP pools were determined in KB and PC-3 cells. Upon the depletion of p53R2 in KB cells, the individual dATP, dTTP, dCTP, dGTP and total dNTP pools in mitochondria decreased modestly from 7.3, 12.6, 21.8, 11.9 and 53.6 to 5.8, 11.4, 20.5, 7.9 and 45.6 pmoles per million cells, respectively (Fig. 2C). In addition, the p53R2 siRNA also exhibited comparable no effects on the whole cell dNTP pools of dATP, dTTP, dCTP, dGTP and total dNTP pools, decreasing from 94.8, 199.8, 300.1, 169.1 and 763.7 to 83.6, 175.5, 291.6, 148.2 and 698.9 pmoles per million cells of KB cells, respectively (Fig. 2D). A similar observation was made in PC-3 cells (Fig. 2E and 2F). Finally, there are no differences between the mock and scrambled siRNA treated cells (Fig. 2C, 2D, 2E, 2F). Consistent with the observation that the RRM1 level is too low, to form functional holoenzyme with p53R2 in mitochondria, the manufacturing dNTPs in the mitochondria is decimal. Consequently, the inhibition of p53R2 expression by si-p53R2 revealed no marked dNTP pool changes, further supporting the notion that p53R2 has negligible impact on both mitochondrial and whole cell dNTP pools in KB and PC-3 cells.

p53R2 affects mitochondrial membrane potential, ATP synthesis, cytochrome c oxidase activity and protects the mitochondrial membrane against oxidative stress in KB and PC-3 cells

JC-1 is a $\Delta\Psi_{mt}$ sensitive probe, forming monomers (green fluorescence) at lower $\Delta\Psi_{mt}$ and JC-1 aggregates (red fluorescence) at higher $\Delta\Psi_{mt}$ [16, 17]. A red fluorescence intensity (RFI) of 300 was used to divide the cells into two populations: a RFI >300 indicating cells with intact mitochondria and a RFI <300 representing cells with defective mitochondria. Based on this criterion, we distinguished cells with intact (designated as "R2" cells) and injured mitochondria (designated as "R1" cells). Fig. 3A showed that the transfection of p53R2 siRNA shifted these RFI toward lower values in KB and PC-3 cells. The percentage of R1 cells increased from 15.1% (Scramble siRNA) to 32.4% (p53R2 siRNA) and 16.1% (Scramble siRNA) to 27.1 % (p53R2 siRNA) in KB and PC-3 cells, respectively. There are no significant differences between the mock- and the scramble siRNA-transfected cells (data not shown). Our results revealed that the knockdown of p53R2 expression caused mitochondrial membrane dysfunction in KB and PC-3 cells.

Since mtDNA content affects mitochondrial function and biogenesis [18], we hypothesize that p53R2 plays a key role in maintaining mitochondria function. To test this hypothesis,

we measured the free ATP synthesis in mitochondria and cytochrome c oxidase activity to probe the mitochondrial function in these cells. After suppressing p53R2 expression by si-p53R2, free ATP amount was significantly reduced to 59.3% and 62.82% of the control ($p < 0.05$) in KB and PC-3, respectively (Fig. 3B). This observation is compatible with the decrease in mtDNA content shown above. Lower mitochondrial cytochrome c oxidase activity after p53R2 knockdown, was also detected after p53R2 suppression in KB and PC-3 cells, decreasing by 88.8% and 87.3% of the control, respectively (Fig. 3C). There were no noticeable differences between the mock and the scramble siRNA-transfected cells (Fig. 3B&3C).

Because mitochondria are particularly susceptible to oxidative damage, leading to mitochondrial membrane potential decrease [17], we investigated the influence of p53R2 on $\Delta\Psi_{mt}$ in response to H_2O_2 in KB-wt, KB-p53R2S and KB-p53R2AS cells with JC-1 staining (p53R2 protein level in these three cells are shown in our previous paper) [15]. The mean fluorescence intensity (MFI) ratio of the red/green ratio was plotted as a function of H_2O_2 concentration (Fig. 4A). KB-p53R2S cells were more resistant to H_2O_2 attack than KB-wt cells, shown by MFI increase, whereas KB-p53R2AS cells were more sensitive to H_2O_2 attack than KB-wt cells with MFI decrease. These results clearly show that p53R2 protected the mitochondrial membrane against oxidative stress. This idea is supported by our previous publication that showed the catalase activity was displayed by recombinant p53R2 protein [15]. Furthermore, intracellular ROS was reduced in p53R2-overexpressing cells compared with control cells in response to H_2O_2 (Fig. 4B). Taken together, p53R2 impacts mitochondrial function, such as membrane potential, ATP synthesis and cytochrome c oxidase activity, and protects mitochondrial membrane against the oxidative stress in KB and PC-3 cells.

Discussion

There are two major findings from this study. First, human KB and PC-3 cancer cells have lower contents of mtDNA after p53R2 knockdown, suggesting that mtDNA contents correlate with the steady-state p53R2 protein level. Second, p53R2 affects mitochondrial membrane potential, ATP synthesis, cytochrome c oxidase activity and protects mitochondrial membrane against oxidative stress. Based on these observations, we propose p53R2 is essential for proper mitochondrial function and homeostasis.

Current paradigm suggests RRM2 associated RR activity is responsible for DNA synthesis, whereas p53R2 participates in DNA repair through synthesizing dNTPs [6]. Loss of p53R2 function causes mtDNA depletion in human [2, 8]. It has been shown that the reduced expression of p53R2 diminishes the level of dNTPs for mtDNA replication and repair, resulting in mtDNA depletion [19, 20]. dNTPs are synthesized by the RRM1/p53R2 complex in the nonproliferating cells [21]. However, it appears that reduced expression of p53R2 renders the mtDNA content depletion and impairs mitochondria function without affecting the whole cell or mitochondria dNTPs in KB and PC3 cells. Thus, unlike in the normal fibroblasts, dNTPs are most likely imported into mitochondria from cytosolic pools in cancer cells [22]. Recently, Ylikallio et al. showed that overexpression of two RR subunits led to imbalanced of dNTP pools and depletion of mtDNA copy number in mouse skeletal muscle [23]. In contrary, we did not see the same phenomena in KB and PC3 cells. The difference may be due to different cells (Cancer cells vs normal cells, oropharyngeal vs muscle, human vs mice) and different assays for mtDNA copy number determination (q-PCR vs Southern blotting). We will determine dNTP pool under different expression level of RR subunits in different cell lines in the future to find out general rule regarding how dNTP pool affects mtDNA integrity.

Our data further point to a novel role of p53R2 in mitochondrial function. We showed overexpression of p53R2 reduced intracellular reactive oxygen species (ROS) and protected mitochondrial membrane potential against oxidative stress. Conversely, ROS level was elevated significantly upon oxidative stress and mitochondrial membrane potential was no longer maintained after p53R2 suppression. A variety of assays demonstrated that mitochondrial functions are retarded when p53R2 level is critically low. These findings are consistent with report that the p53R2-knockout mouse embryo fibroblasts (MEFs) exhibit greater sensitivity to H₂O₂ insult than their corresponding wild-type MEFs [2]. Together with our previous reports that p53R2 Ser-72 phosphorylation by ATM is refractory to MDM2-dependent degradation of [24] and that p53R2 is dissociated from p21 at G1 phase upon the exposure of UV [25], we postulate a new paradigm that knockdown p53R2 triggers ROS accumulation and further perturbs ATM and MDM2 in DNA repair and cell cycle regulation. As a consequence of increased in ROS, the mitochondria membrane is got impaired and mitochondria function is deteriorated. In our current study, free ATP synthesis decreased significantly upon the depletion of p53R2 in KB and PC-3 cells. It has been well established that the ATP is immediately and significantly decreased from damaged mitochondria [26].

It has been postulated that mitochondrial defects play an important role in the development and progression of cancer [27, 28]. Studies have shown correlation between mitochondrial defects and cancer; defects including altered expression, activity of respiratory chain subunits, glycolytic enzymes and mtDNA mutations. Mutations and deletions in mtDNA and abnormal expression of mtDNA-encoded proteins have been observed in various solid tumors and hematological malignancies [27–30]. We have previously reported that p53R2 directly interacts with wild-type p53 and dissociated from p53 under UV stress, and the dissociated p53R2 forms complex with RRM1 to provide dNTPs for DNA repair in the nucleus [31]. By contrast, the UV-induced p53R2 transcription and binding of p53R2 with RRM1 are impaired in p53 truncated PC-3 cells [10]. In this study, we found that inhibition of mitochondrial function by p53R2 in PC-3 cells is comparable to that in KB cells. This finding suggests that the activity of p53R2 in maintaining mitochondria function might not depend on functional p53. It is not clear whether p53R2 have differential effect on antagonizing ROS and the downstream events in KB and PC-3 cells. The answers to these questions would undoubtedly advance our understanding of p53R2 and mitochondrial biology in cancer, as well as provide a basis for designing new strategies for effective cancer prevention, diagnosis, and treatment.

In summary, we used complementary genetic, cell biological and biochemical approaches to underscore the critical role of p53R2 in mitochondria homeostatic control in cancer cells. Our findings provide previously uncharacterized mechanistic insights into how p53R2 impacts the mitochondria integrity, influencing not only the mtDNA content, but also the mitochondria function. The work presented expands the concept of how p53R2 functions, and how it participates in cellular responses to ROS in cancer cells.

Highlights

>We revealed that p53R2 expression positively correlates with mtDNA content. >We found p53R2 expression impacts the mitochondrial function. >Overexpression of p53R2 reduces ROS and protects mitochondrial membrane potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. p53R2 expression level is associated with mtDNA content in KB and PC-3 cells

A, q-RT-PCR analyses reveal that the steady-state p53R2 mRNA level decreased by ~90% in KB and PC-3 cells at 48 h post transfection with p53R2 siRNA. B, Western blotting analyses illustrate that the p53R2 protein amount was significantly downregulated in KB and PC-3 cells at 48 h post transfection with si-p53R2. C, Quantification of the mtDNA using real-time PCR to assess mitochondrial gene *COX-I* amount relative to that of the *PDE6B* nuclear gene. D, Quantitative PCR analyses of mitochondrial gene *ND1* amount to estimate mtDNA content relative to the β -*GLOBIN* nuclear gene. Data shown as the mean $\pm$ S.D. calculated from at least 3 independent experiments. * depicts $p < 0.05$.

Fig. 2. Inhibiting the expression of p53R2 has modest effect on mitochondrial dNTP pools in KB and PC-3 cells

A, RR activity in cytosol extracts (CE) and mitochondrial extracts (ME) in KB and PC-3 cells is shown. Data presented as means of three separate experiments with duplicate. B, Western blot analysis of RRM1 in CE and ME. The presence of RRM1, COXIV or GAPDH was visualized using antibody against the corresponding protein. C to F, Mitochondrial (C and E) and whole cell (D and F) individual dNTP and total dNTP pools in KB (C and D) and PC-3 (E and F) cells at 48 h post transfection with p53R2 siRNA, scramble siRNA or mock cell are shown. Data are shown as the mean $\pm$ S.D. from at least 3 independent experiments.

Fig. 3. p53R2 inhibited by siRNA decreases the mitochondrial membrane potential, ATP synthesis and cytochrome C oxidase activity in KB and PC-3 cells

A, Histogram of JC-1 staining of KB and PC-3 cells transfected with scramble or p53R2 siRNA. The JC-1 red fluorescence intensity (RFI) depicts $\Delta\Psi_{mt}$. R1 cells: RFI <300 (injured mitochondria) and R2 cells: RFI >300 (intact mitochondria). The data shown here is one representative out of 2 to 3 independent experiments. B, Relative ATP levels in KB and PC-3 cells with or without p53R2 siRNA. The level of ATP in cells transfected with scramble siRNA is designated at 100%. Bar represents mean $\pm$ S.D. of at least 3 independent experiments, * indicates $p < 0.05$ (p53R2 vs scramble siRNA). C, Inhibition of mitochondrial cytochrome c oxidase activity by p53R2 siRNA at 48 h post transfection. Data are presented as the mean $\pm$ SD from at least 3 independent experiments, * indicates $p < 0.05$ (p53R2 vs scramble siRNA).

Fig. 4. p53R2 protects mitochondrial membrane from H₂O₂ attack and reduces intracellular ROS

A, KB-wt, KB-p53R2S and KB-p53R2AS cells treated with 250 μmol/L of H₂O₂ for 24 hours, were stained with 1 μg/mL JC-1 for 30 minutes at 37°C before harvesting. The variations of the red/green mean fluorescence intensity ratio are as a function of the H₂O₂ concentration. * indicates $p < 0.05$. B, KB-vector and KB-p53R2S cells were treated with indicated concentrations of H₂O₂ for 24 hours before staining with 10 μmol/L carboxy-H2DCFDA. Carboxy-DCF generation (FI) was monitored fluorometrically. Data shown are the mean $\pm$ SD based on three independent experiments, each carried out in quadruplicate.