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Mitochondrial protein OPA mediates osteoporosis induced by radiation through the P38 signaling pathway

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Abstract. – OBJECTIVE: Bone marrow is full of mitochondria. However, the role of bone marrow mitochondrial protein in bone marrow damage and related signal transduction mechanism remains to be further studied. OPA is a newly discovered mitochondrial transmembrane protein. Its expression pattern and function in the physiological and pathological conditions of bone marrow are still elusive. The purpose of this study is to investigate the potential role of OPA in osteoporosis.

PATIENTS AND METHODS: A mouse osteoporosis model was established by radiation. The OPA expression was tested by Western blot and qRT-PCR. The P38 signaling activity was evaluated by enzymatic activity kit. The mitochondrial ATP production was determined by flow cytometry. The bone marrow cell apoptosis was detected by flow cytometry. U0126 was used to pretreat mouse before modeling. Bone marrow tissue was collected from patients who received osteoporosis surgery to test the OPA expression, P38 activation and cell apoptosis. The OPA and P38 levels were analyzed by correlation.

RESULTS: The mouse osteoporosis model was successfully established by radiation induction. In this osteoporosis model, the expression of OPA was increased. The P38 signaling was activated while the mitochondrial ATP production was reduced, with the increase of apoptosis of bone marrow cells. By contrast, U0126 pretreatment markedly inhibited the OPA expression, restrained the P38 signaling pathway, enhanced mitochondrial ATP production and suppressed the bone marrow cell apoptosis in mouse osteoporosis model. A significantly positive correlation was found between OPA and P38.

CONCLUSIONS: The down-regulation of OPA inhibits cell apoptosis and improves osteoporosis via inducing mitochondrial ATP production and suppressing the P38 signaling pathway.

Key Words:
Mitochondrial protein OPA, P38 signaling pathway, Radiation, Osteoporosis.

Introduction

Osteoporosis is a set of bone disease caused by a variety of reasons. Normal calcification in bone tissue maintains the proportion of calcium salt and matrix. Osteoporosis is characterized by a bone amount reduction in unit volume. The formation reasons include hypercortisolism, pregnancy, lactation, protein deficiency, vitamin C and D deficiency, osteopsathyrosis chromosomal abnormalities, and radioactivity produced by high voltage and ionizing radiation. The osteoporosis induced by radiation is a bone marrow inflammatory response syndrome caused by infection, which is featured as high incidence, fatality rate, cost and rising incidence[1,2]. The osteoporosis caused by radiation may induce tissue hypoperfusion and septic shock through multiple mechanisms, leading to organ dysfunction and death[3-5]. Therefore, the detection of osteoporosis in mice is the premise and foundation of osteoporosis treatment caused by radiation[6,7]. According to the current standard of osteoporosis caused by radiation, the mice suffered from severe inflammation when diagnosed, which interferes the efficacy of treatment[8]. The biomarkers with good specificity and sensitivity

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are of great significance for the early diagnosis, treatment and prognosis of osteoporosis caused by radiation. The previous study on the biomarker for osteoporosis caused by radiation mainly focused on inflammatory factors and indicators concerning bone marrow organizational dynamics. However, these biomarkers exhibited poor specificity and sensitivity[9,10]. Thus, ideal biomarkers for osteoporosis are still urgently needed[11].

Osteoporosis caused by radiation involves a variety of mechanisms, including inflammatory mediator-induced mitochondrial structure and function changes, namely mitochondrial dysfunction[12,13]. Mitochondria are abundant in bone marrow cells, which supply uninterruptible energy. Myocardial mitochondria are an important target of bone marrow cell damage in osteoporosis caused by radiation. It is speculated that mitochondrial energy metabolism is associated with dynamics change[14,15]. Mitochondrial protein OPA plays a critical role in mitochondrial function[16], which may be closely related to osteoporosis[17,18]. The relationship between OPA and osteoporosis caused by radiation remains to be elucidated[19]. This study intends to explore the role of OPA in osteoporosis with the mouse model.

Patients and Methods

Reagents and Methods

FBS and cell medium was purchased from Hualan Biological (Beijing, China). Fluorescein isothiocyanate (FITC)-annexin and caspase-3 detection kits were bought from Beyotime (Beijing, China). Protein extraction kit and bicinchoninic acid (BCA) protein detection kit from Solarbio (Beijing, China). Horseradish peroxidase (HRP) labeled rabbit anti-mouse IgG, mouse anti-human OPA monoclonal antibody, and actin monoclonal antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Experimental Model

The mouse osteoporosis model was established by radiation according to the reference[10]. The mice were bought from Beijing HFK Bioscience co., Ltd (Beijing, China) and treated by 25 μg/kg radiation. The study was approved by the Ethics Committee of The 2nd Hospital, Medical College, Shantou University (Shantou, China) and conformed to animal welfare. The bone marrow tissue was collected from osteoporosis patients in our hospital with mean age 46.2 ± 4.7 (32-56 years old). Normal bone marrow was extracted from healthy volunteers with the mean age 48.5 ± 3.2 (34-54 years old). No statistical difference was observed on age between the two groups (p=0.014). This study was approved by the Ethics Committee of The 1st Hospital, Medical College, Shantou University (Shantou, China) and all the subjects had provided an informed consent.

Bone Marrow Tissue Sample Preparation

The bone marrow tissue was extracted from mice or patients and cracked to prepare the suspension. The suspension was mashed by normal saline and acetone four times. Next, the sample was centrifuged at 150 g for 6 min and stored at -20 °C.

Caspase-3 Activity Analysis

The P38 signaling activity was detected by enzymatic activity detection kit. Bone marrow cells were collected using the routine method[22,23]. Caspase-3 activity was evaluated on a microplate reader (Bio-Rad, Hercules, CA, USA)[24]. A total of 2×10^5 bone marrow cells were added with lysate to extract protein. Then the lysate was incubated in the caspase-3 chromophoric substrate in the dark at room temperature for 20 min. At last, the samples were tested at 492 nm on a microplate reader (Bio-Rad, Hercules, CA, USA).

Adenosine 5'-Triphosphate (ATP) Production Detection

Flow cytometry was applied to test mitochondrial ATP production in bone marrow cells or tissues. A total of 30 μg sample was added with 150 μl lysate to prepare the suspension. Next, the sample was added with detection liquid and tested at 560 nm on a microplate reader (Bio-Rad, Hercules, CA, USA). The ATP concentration was calculated based on a standard curve.

Western Blot

Western blot was used to test the OPA expression in bone marrow. The protein was extracted from the bone marrow sample and quantified by BCA kit. A total of 15 μg protein was boiled for 7 min and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After being transferred to the membrane at 140 mA for 2 h, the membrane was blocked by 8% skim milk at room temperature for 1.5 h. Next, the membrane was incubated in OPA, P38 or actin monoclonal antibodies (1:1000) at 4 °C overnight. Then, the membrane was washed with Tris-Buffe-

red and Tween 20 (TBST) solution and incubated in the secondary antibody (1:1500) at 37°C for 2 h. At last, the membrane was developed by enhanced chemiluminescence (ECL) and imaged on gel imaging system (Shanghai Qianxiang, Shanghai, China) to analyze the protein expression.

Real Time-PCR

Total RNA was extracted from the sample by TRIzol (Invitrogen, Carlsbad, CA, USA) and reversed transcribed to cDNA for Real Time-Polymerase Chain Reaction (PCR)[22]. Especially, poly A was used as a primer for reverse transcription, which was performed at 42°C for 60 min. PCR reaction system contained 1 µl cDNA, 2 µl primers, 4 µl dNTP, 2 µl buffer and 1 µl Taq enzyme. The PCR reaction was performed at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s and 55°C for 30 s. The primers used were as follows. OPA 5'-TTATTAGAGGGTGGGGTGGATTGT-3' and 5'- CCACCTAAATCAACCTCCAACCA-3'. Actin 5'-TTATTAGAGGGTGGGGCGGATCGC-3' and 5'- CCACCTAAATCGACCTCCGACCG-3'.

Caspase-3 Activity Detection

The bone marrow cells were resuspended in Dulbecco's Modified Eagle's Medium (DMEM) and added with the chromophoric substrate. Next, the cells were tested on a microplate reader to obtain the absorbance value[17]. Caspase-3 relative activity was calculated based on the absorbance value of the cell treated by radiation and control.

U0126 Pretreatment

To further analyze the impact of the P38 signaling pathway in osteoporosis caused by radiation, the P38 signaling inhibitor U0126 (10 µg/kg) was intraperitoneally injected to mice. Next, the mice were used to establish osteoporosis caused by radiation[10]. The bone marrow was extracted for Western blot, ATP production and cell apoptosis detection.

Statistical Analysis

All data analyses were performed on SPSS 11.0 software (SPSS IBM, Armonk, NY USA). Data were presented as means ± standard deviation (SD). Those samples fitted normal distribution were compared by two-sample independent *t*-test. One-way ANOVA, with Tukey's post-hoc test was performed for the analysis of data from multiple groups. $p<0.05$ was considered statistically significant.

Results

OPA mRNA and Protein Increased in Bone Marrow Cells From Osteoporosis Mice

As shown in Figure 1A, qRT-PCR data exhibited that OPA mRNA expression was significantly elevated in bone marrow cells from osteoporosis mice compared with that in normal control ($p<0.05$). As shown in Figure 1B and C, the OPA protein level was also markedly upregulated in bone marrow cells from osteoporosis mice compared with that in normal control ($p<0.05$).

P38 Signaling is Activated, Mitochondrial ATP Production is Reduced While Cell Apoptosis is Enhanced in Bone Marrow Cells From Osteoporosis Mice

To further analyze the mechanism of OPA on osteoporosis caused by radiation, we detected mitochondrial ATP production and cell apoptosis. As shown in Figure 2, Western blot results demonstrated that, in the osteoporosis model caused by radiation, the P38 signaling was remarkably acti-

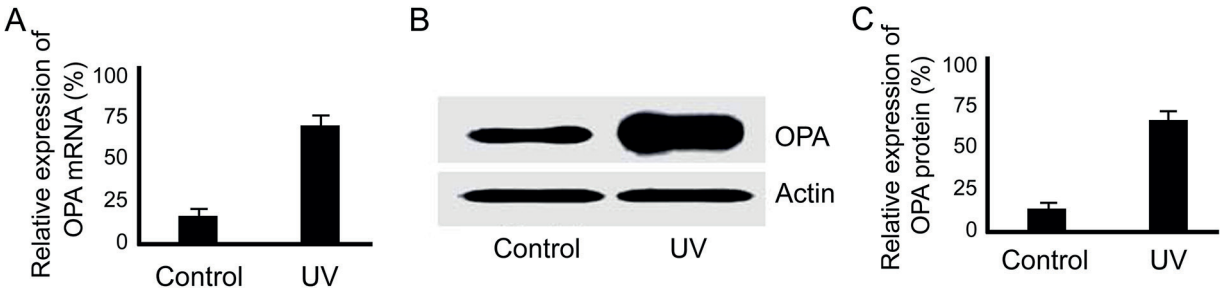

Figure 1. OPA expression in bone marrow cells from osteoporosis mice. ***A,*** qRT-PCR detection. ***B,*** Western blot detection. ***C,*** Western blot analysis. *$p<0.01$, compared with control.

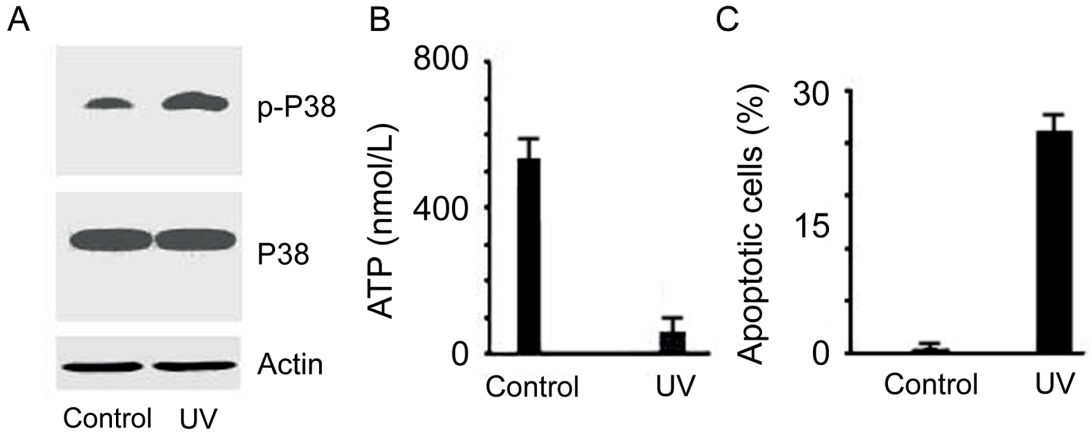

Figure 2. P38 signaling is activated, mitochondrial ATP production is reduced, while cell apoptosis is enhanced in bone marrow cells from osteoporosis mice. ***A,*** Western blot detection. ***B,*** ATP production analysis. ***C,*** Cell apoptosis analysis. *$p<0.01$, compared with control.

vated. Moreover, mitochondrial ATP production was significantly decreased, while cell apoptosis was statistically enhanced in bone marrow cells compared to that in the control group ($p<0.05$).

The Impact of P38 Signaling Inhibitor U0126

We further investigated the change of osteoporosis after the P38 signaling pathway was inhibited by U0126. Of note, Western blot revealed that U0126 pretreatment markedly suppressed the OPA expression, restrained the P38 signaling activation and enhanced mitochondrial ATP production. Caspase-3 activity detection exhibited that U0126 pretreatment significantly inhibited bone marrow cell apoptosis (Figure 3). It suggested that the P38 signaling played an important role in osteoporosis caused by radiation.

OPA and Cell Apoptosis Detection in Bone Marrow From Osteoporosis Patients

To further determine the OPA expression, the P38 signaling pathway and cell apoptosis in osteoporosis patients, we collected bone marrow tissue from osteoporosis patients. As shown in Figure 4, OPA level was upregulated, P38 was activated and cell apoptosis was enhanced in bone marrow tissue from osteoporosis patients.

Correlation Analysis of OPA and P38 Levels in Bone Marrow From Osteoporosis Patients

The correlation analysis showed that the OPA expression was positively correlated with the P38 level in bone marrow from osteoporosis patients ($p<0.05$) (Figure 5).

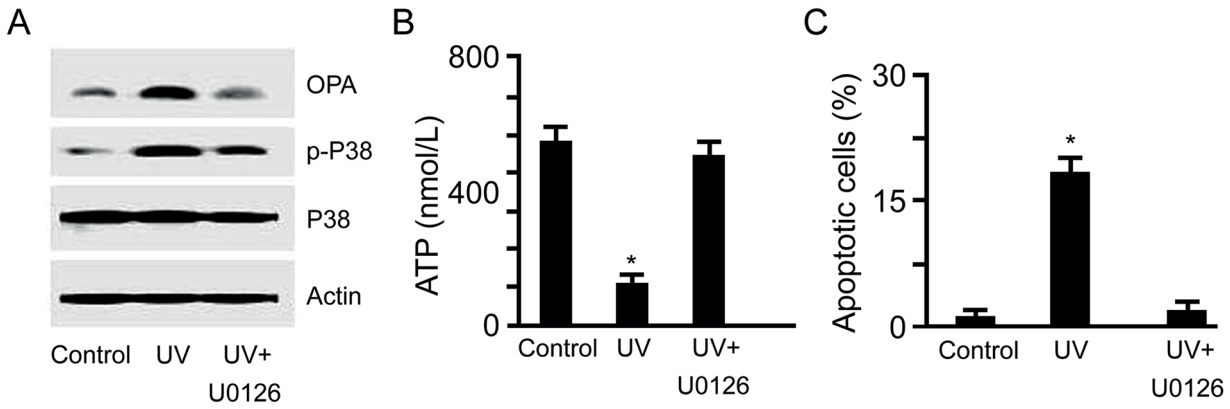

Figure 3. The impact of P38 signaling inhibitor U0126 pretreatment. ***A,*** Western blot detection. ***B,*** ATP production analysis. ***C,*** Cell apoptosis analysis. *$p<0.01$, compared with control.

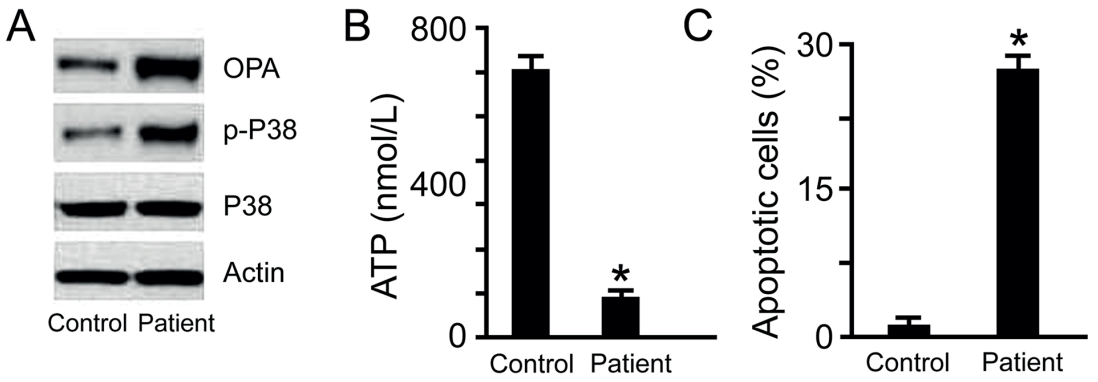

Figure 4. OPA and cell apoptosis detection in bone marrow from osteoporosis patients. ***A,*** Western blot detection. ***B,*** ATP production analysis. ***C,*** Cell apoptosis analysis. *p<0.01, compared with control.

Discussion

Osteoporosis caused by radiation is a type of bone marrow inflammatory response syndrome induced by infection, which is a fatal threat to the mice. The osteoporosis caused by radiation may induce septic shock through various mechanisms, leading to organ dysfunction and death. Mitochondrial uncoupling protein (OPA) regulates inflammation reaction, restrains oxidative stress and maintains the balance of mitochondrial membrane potential and energy production. The role of OPA in the osteoporosis caused by radiation remains to be further discussed.

Accumulative evidence indicated that it was extremely important for the early diagnosis and treatment of osteoporosis caused by radiation[25,26]. However, it still lacks of effective biomarkers for the detection of osteoporosis caused by radiation in clinic[27]. Thus, this study discussed the possibility of OPA as a molecular biomarker for the early diagnosis and prognosis of osteoporosis caused by radiation. Our results suggested that OPA was upregulated, the P38 signaling was activated, mitochondrial ATP production was reduced and bone marrow cell apoptosis was enhanced in the osteoporosis model. U0126 pretreatment suppressed the OPA expression, restrained P38 signaling, enhanced mitochondrial ATP production and inhibited bone marrow cell apoptosis induced by radiation, indicating that OPA is in favor of osteoporosis diagnosis, which was in accordance to previous studies[25,26]. Osteoporosis is a highly complex inflammation process in the clinic. A variety of inflammatory factors interact, resulting in immune response activation, which is one of the most important causes of osteoporosis[1,2]. Our results observed that, according to the current diagnosis standard for osteoporosis caused by radiatio[20,21], the immune response became potent even in a general state of osteoporosis, leading to a great threat to life and health. The mitochondrial abnormalities and state disorder were also found in this work.

The molecular mechanism of OPA in osteoporosis is still unclear. At present, there is still a lack of report about OPA as a biomarker for the early diagnosis of osteoporosis caused by radiation. This study mainly elaborated the mechanism of osteoporosis. Our results showed that OPA and P38 exhibited a positive correlation. OPA affected mitochondrial ATP production and cell apoptosis in mice osteoporosis model caused by radiation through the mediation of P38 signaling, which was similar to previous studies[25,26]. Other researches demonstrated that radiation produced reactive oxygen species (ROS) in mice through sustained activating inflammation, including immune-related factors[14]. In addition, ROS may upregulate inflammatory factors[15,16]. Thus, inflammatory factors can regulate redox signal, while their roles as a target for osteoporosis are still unclear. The relationship between OPA and this signaling still needs further

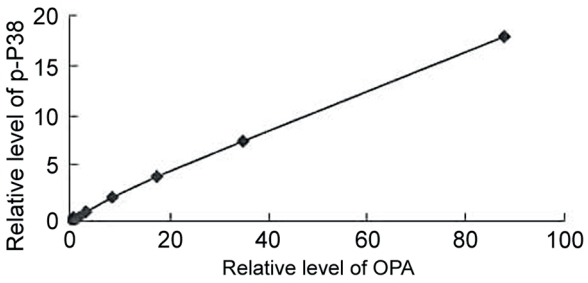

Figure 5. Correlation analysis of OPA and P38 levels in bone marrow from osteoporosis patients.

investigation within a large size of samples. Moreover, the influence of chemotherapy received by osteoporosis patients on OPA expression requires further elucidation[27,28]. Additionally, novel targeting drugs, such as rutin, ought to be developed with a definite evaluation of efficacy, based on the experimental data[29].

Conclusions

Our data demonstrated that the level of OPA is involved in the bone marrow of mice osteoporosis model and clinical osteoporosis patients caused by radiation. It regulates mitochondrial ATP production and cell apoptosis through the P38 signaling, which provides a theoretical basis for OPA as a biomarker in early diagnosis and prognosis of osteoporosis caused by radiation.

Acknowledgments

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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