Inhibition of centrosomal protein 164 sensitizes rhabdomyosarcoma cells to radiotherapy

JIANZHOU LIU¹, ZHIJU WANG², XIAOFENG LI¹, XU ZHANG¹ and CHAOJI ZHANG¹

¹Department of Cardiac Surgery, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences, Beijing 100730; ²Department of Physiology, School of Basic Medicine Science, Zhengzhou University, Zhengzhou, Henan 450001, P.R. China

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Abstract. Rhabdomyosarcoma is the second most common malignant tumor of the heart in infants and children and cannot often be resected completely. Chemotherapy and radiotherapy have a critical role in relieving symptoms and prolonging survival; therefore, enhancing the sensitivity of rhabdomyosarcoma to radiotherapy is an important area of investigation in order to improve the prognosis of patients. It has been reported that centrosomal protein 164 (CEP164) has a key role in the DNA damage-activated signaling cascade. CEP164 is often overexpressed in tumors and is associated with poor prognosis in various types of cancer. In the present study, the influence of CEP164 on the radiosensitivity of rhabdomyosarcoma cells was investigated. Results demonstrated that CEP164 is involved in the radiation-induced cellular response. CEP164 is increased upon radiation and influences the cell cycle, cell viability and cell apoptosis. CEP164 depletion enhanced cellular sensitivity to radiation, promoted cell apoptosis, decreased cell viability and induced gap 2/mitosis arrest of the cell cycle. The present study identified the function of CEP164 in radiation resistance in rhabdomyosarcoma, providing a potential therapeutic target for rhabdomyosarcoma treatment by disrupting CEP164.

Introduction

The heart is an uncommon organ for primary tumor development. Primary rhabdomyosarcoma of the heart is a rare tumor; however, it is the second most common cardiac sarcoma (1). Rhabdomyosarcoma arises in the right atrium and is often complicated with heart failure, which is serious and difficult to control (2). Early recognition and therapeutic intervention is

E-mail: cjzhang_pumc@163.com

extremely important in the prevention of heart failure. Surgery is the mainstay of treatment for non-metastatic disease. If surgical intervention fails to completely resect the tumor (3,4), radiotherapy is essential and effective; however, the therapeutic success of chemotherapy and radiotherapy is poor for metastatic disease (5,6). Therefore, the improvement of radiotherapy success for rhabdomyosarcoma has become a major focus of research.

Proteins of the centrosomal protein (CEP) family are critical components of centrosomes and have vital roles in the control of cell cycle progression. This protein family consists of 31 proteins, one of which is CEP164. CEP164 encodes a 180 kDa protein. CEP164 may be phosphorylated by ataxia telangiectasia mutated (ATM) kinase and Rad3-related protein (ATR) kinase in the DNA damage response (DDR) pathway and has a key role in the gap 2/mitosis (G2/M) checkpoint (7,8). Research has demonstrated that mutations in CEP164 are one cause of nephronophthisis-related ciliopathies (NPHP-RC) (9). In the DDR, CEP164 is rapidly localized to nuclear foci. Research has demonstrated that knockdown of CEP164 induces cell sensitivity to DNA-damaging agents and CEP164 knockdown in zebrafish results in deregulated DDR and an NPHP-RC phenotype (4). Additionally, CEP164 modulates mediator of DNA damage checkpoint protein 1 (MDC1) and checkpoint kinase 1 (CHEK1) to maintain genomic stability (10).

The importance of CEP164 in the DDR makes it a notable target for enhancing the radiosensitivity of cells; however, the effect of combining CEP164 inhibition with irradiation (IR) in rhabdomyosarcoma cells is unknown. In the present study, the role of CEP164 in the cell cycle, and the cell viability of the rhabdomyosarcoma A-204 and A-673 cells lines following treatment with IR were investigated. It was demonstrated that CEP164 expression levels are increased following treatment with IR and depletion of CEP164 enhances cellular sensitivity to radiation, resulting in decreased cell viability, promotion of apoptosis and G2/M cell cycle arrest. In conclusion, the present study demonstrated that CEP164 may be a candidate target gene for rhabdomyosarcoma radiotherapy.

Materials and methods

Cell culture, small interfering (si)RNA transfection and radiation exposure. Human rhabdomyosarcoma A-204 and A-673 cells (American Type Culture Collection, Manassas, VA, USA)

Correspondence to: Professor Chaoji Zhang, Department of Cardiac Surgery, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences, 1 Shuaifuyuan, Dongdan, Beijing 100730, P.R. China

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were cultured in McCoy's 5A (Modified) Medium supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), incubated at 37°C in a 95% humidified incubator (5% CO₂) and irradiated with doses of 2, 4 or 6 Gy. Cells were treated with Cobalt-60 γ -rays at 2 Gy/min in the Beijing Radiation Center (Beijing Academy of Science and Technology, Beijing, China). Cells were cultured to 80% confluence and were subsequently transfected with siRNA against the negative control or against CEP164 (Ambion; Thermo Fisher Scientific, Inc.). All transfection was performed using Lipofectamine[®] RNAiMAX reagent (Thermo Fisher Scientific, Inc.). Cells were exposed to IR 24 h after target gene knockdown.

Western blot analysis. A-204 cells were lysed in RIPA lysis and extraction buffer containing protease and phosphatase inhibitor (all Pierce; Thermo Fisher Scientific, Inc.) for 20 min at 4°C. Protein concentration was measured using a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. A total of 30 μ g of protein was separated by 10% Bis-Tris gels (Thermo Fisher Scientific, Inc.) and transferred onto nitrocellulose membranes for western blot analysis. Following washing three times with Tris-buffered saline-Tween 20, the membranes were blocked with 5% nonfat milk for 60 min and were subsequently incubated with primary anti-CEP164 (1:1,000; GTX85298; GeneTex, Inc., Irvine, CA, USA) or anti-β-actin antibodies (1:1,000; ab8227; Abcam, Cambridge, UK) overnight at 4°C. Following two washes with washing buffer (0.5% Tween in PBS), the membranes were incubated with horseradish peroxidase-conjugated secondary anti-rabbit antibody (1:3,000; ab6721; Abcam) for 1 h at room temperature. Following this, protein bands were detected using chemiluminescence liquid (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions, and analyzed using ImageJ software (ImageJ 2x version 2.1.4.6, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell cycle assay. A total of $1x10^6$ cells were harvested and resuspended in 1 ml McCoy's 5A (Modified) Medium supplemented with 10% FBS. Cells were stained with 2 μ l Vybrant DyeCycle Green Stain and incubated at 37°C for 30 min. The cell cycle was analyzed using flow cytometry using 488 nm excitation and green emission.

Cell viability assays. Following treatment with IR, cells were mixed with 10% volume of alamarBlue reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated for 30 min at 37°C, protected from direct light. Results were recorded using florescence at 570/585 nm (excitation/emission).

Cell apoptosis assay. Cells were harvested and washed twice with cold phosphate-buffered saline. Subsequently, cells were resuspended in 1X binding buffer (BD Biosciences, San Jose, CA, USA) at a concentration of 1x10⁶ cells/ml and stained with PE Annexin V with 7-AAD (BD Biosciences) in the dark for 15 min at room temperature. Samples were analyzed using flow cytometry.

Statistical analysis. Statistical analysis was performed using Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA).

Data are presented as the mean \pm standard error of the mean of three independent experiments. Differences in mean values between groups were determined by Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

 γ H2AX foci formation. A-204 cells were seeded onto glass slides. Following transfection with siRNAs, the cells were irradiated with 4 Gy for 30 min, washed twice in PBS for 5 min and subsequently fixed for 10 min at room temperature using 4% paraformaldehyde. Cells were washed twice in PBS for 5 min, permeated for 10 min at room temperature using permeabilisation buffer (0.2% TritonX-100 in PBS) and washed twice more for 5 min. Cells were subsequently blocked for 1 h at room temperature with blocking buffer (0.05% FBS in PBS), and incubated with anti-yH2AX (1:200; Merck KGaA, Darmstadt, Germany; 05-636-AF488) antibody overnight at 4°C. The cells were washed twice more in washing buffer (0.05% Tween in PBS) and the coverslips were mounted with mounting medium (glycerol), stained with DAPI and detected using a fluorescent microscope (Leica Microsystems GmbH, Wetzlar, Germany). yH2AX foci were analyzed using Image Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Results

Radiation induces CEP164 protein expression. To determine whether CEP164 is involved in the DDR, cells were γ -irradiated with 2, 4, 6 Gy and, following this, CEP164 expression levels were measured. Results demonstrated that CEP164 protein expression levels increased following IR with 2 and 4 Gy and the expression levels peaked at 24 h following treatment with 2 Gy IR in A-204 and A-673 cells, respectively (Fig. 1).

CEP164 inhibition induces G2/M arrest of the cell cycle. In order to determine whether CEP164 has a regulatory role in cell cycle progression, siRNA was utilized to knockdown CEP164 expression levels in A-204 and A-673 cells and, following 24 h, G2/M cell cycle arrest was measured. Results demonstrated that CEP164 delayed S-phase exit. Furthermore, to determine whether inhibition of CEP164 enhances the radiosensitivity of A-204 and A-673 cells, the cell cycle of CEP164-depleted cells following treatment with IR was analyzed. Treatment of A-204 cells with 2 Gy of IR induced entry into G2/M phase and a lower population in the synthesis (S) phase; however, combined radiation and CEP164 inhibition resulted in G2/M cell cycle arrest. These experiments were also conducted on A-673 cells, and similar results were found in CEP164 silencing A-673 cells (Fig. 2).

CEP164 silencing increases cell apoptosis and decreases cell viability upon IR. Following the result that CEP164 inhibition is able to arrest cells in the S phase of the cell cycle, colony formation assays were conducted in order to determine whether CEP164 has a role in cell proliferation and apoptosis. Cell viability assays indicated that CEP164 knockdown significantly decreased cell viability following treatment with IR compared with the negative control IR-treatment group (P<0.05). In order to determine whether CEP164 knockdown enhances IR-induced apoptosis, four different experimental conditions were investigated, including: No IR and no CEP164



Figure 1. CEP164 protein expression levels increase following IR. (A) Western blot analysis of A-204 and A-673 rhabdomyosarcoma cells treated with IR of 2 and 4 Gy. (B) A-204 and A-673 rhabdomyosarcoma cells were exposed to 2 Gy IR and subsequently incubated for 4, 8, 24 and 48 h. CEP164, centrosomal protein 164; IR, irradiation.



Figure 2. CEP164 deletion results in G2/M cell cycle arrest. (A) A-204 and A-673 rhabdomyosarcoma cells were transfected with siCEP164 RNA and subsequently treated with IR. Cell cycle progression was determined by DyeCycle staining. (B) Quantification of cell cycle assays. Similar results were obtained in three experiments. **P<0.01 vs. the negative control group. (C) Western blot analysis of CEP164 expression in CEP164-knockdown cells with or without IR (2 Gy). Data are presented as the mean + standard error of the mean. CEP164, centrosomal protein 164; IR, irradiation; si, small interfering; G0/G1, resting/gap 1; S, synthesis; G2/M, gap 2/mitosis.



Figure 3. CEP164 silencing decreases cell viability and promoted apoptosis upon IR. (A) Relative cell viabilities of A-204 rhabdomyosarcoma cells were measured using alamarBlue staining. (B) siCEP164 transfected A-204 cells were exposed to 4 Gy of IR and cell apoptosis was subsequently quantified. Similar results were obtained in the three experiments. Data are presented as the mean + standard error of the mean. *P<0.05, **P<0.01 vs. the negative control group. CEP164, centrosomal protein 164; IR, irradiation; si, small interfering.

knockdown; IR only (4 Gy); CEP164 knockdown only; and combined CEP164 knockdown and IR (4 Gy). Results demonstrated that the combined treatment of CEP164-silencing and IR produced a significant increase in apoptosis compared with either of the treatments alone (P<0.05, P<0.01; Fig. 3). These results indicate that CEP164 is able to facilitate cellular proliferation and inhibit radiation injury.

CEP164 knockdown upregulates IR-induced γ H2A histone family member X (γ H2AX) foci formation. In order to confirm whether CEP164 is able to enhance the DDR, the γ H2AX foci assembly in A-204 cells with or without CEP164 silencing was investigated. It was demonstrated that CEP164 depletion significantly increased γ H2AX foci assembly compared with the negative control cells during 4 Gy IR (P<0.05), indicating that CEP164 repression may enhance the recruitment of γ H2AX and its associated repair proteins to DNA damage sites (Fig. 4).

Discussion

Cardiac tumors are classified as primary benign and malignant tumors if they arise in the heart, or as secondary tumors if they metastasize to the heart. In children, 90% of cardiac tumors are benign, whereas in adults 75% of cardiac tumors are benign (1,11-13). Rhabdomyosarcoma is the most common soft tissue malignant tumor in infants and children (14). Surgical resection of rhabdomyosarcoma is technically challenging and many malignant tumors cannot be resected completely. Chemotherapy and radiation therapy are able to relieve symptoms and prolong survival. A multi-treatment approach, including chemotherapy, radiation and novel,



Figure 4. γ H2AX foci formation. A-204 cells were stained with anti-phospho histone H2AX (Ser139) alexa fluor 488 conjugate antibody and observed using a fluorescent microscope (magnification, x400). (A) Left panel presents A-204 rhabdomyosarcoma negative control cells without IR treatment and the right panel indicates siCEP164 A-204 rhabdomyosarcoma transfected cells without IR treatment. (B) Left panel presents A-204 rhabdomyosarcoma negative control cells without CEP164 knockdown that were exposed to 4 Gy IR and the right panel indicates A-204 rhabdomyosarcoma cells with CEP164 knockdown that were exposed to 4 Gy IR. (C) γ H2AX foci formation. The number of foci per nucleus were quantified. Data are presented as the mean + standard error of the mean for three independent experiments, *P<0.05 vs. the negative control group. γ H2AX, γ H2A histone family member X; IR, irradiation; siCEP164, small interfering centrosomal protein 164.

evolving approaches, such as gene therapy, may provide a more effective palliative and curative result.

CEP164 protein expression levels and activity are up-regulated in various types of cancer, and the protein is often overexpressed in tumors and is associated with poor prognosis (10). Additionally, CEP164 has a key role in primary cilia formation, which is a marker for distal appendages on mature centrioles or basal bodies (3,6). As a novel mediator protein in DDR, CEP164 is required for the DNA damage-activated signaling cascade (4,10). CEP164 interacts with both ATR and ATM, which are able to phosphorylate CEP164 under conditions of DNA damage and replication stress. Following phosphorylation, CEP164 is able to phosphorylate H2AX and CHEK2 (15). In the present study, the association of CEP164 knockdown with radiosensitivity in the cells was investigated. Results demonstrated that CEP164 protein expression levels increased following IR and inhibition of CEP164 resulted in the increased radiosensitivity of rhabdomyosarcoma cells. Furthermore, the results demonstrated that CEP164 promotes cell survival and decreases cell viability upon IR, indicating that CEP164 has an important role in cellular proliferation in response to cellular stress.

CEP164 is required for genomic stability and CEP164 expression is cell cycle stage-dependent, being expressed at the end of the S phase and the beginning of the G2/M phase (16). CEP164 is essential for G2/M checkpoint activation through the phosphorylation of CHEK proteins. The results of the present study indicated that CEP164 engaged in cellular G2/M arrest, demonstrating that CEP164 has a function in cell cycle switching.

In conclusion, the present study demonstrated that CEP164 deletion is involved in the IR-induced cellular response and enhances the radiosensitivity of rhabdomyosarcoma cells. This, therefore, indicates that CEP164 may be a potential target to improve the outcome of radiotherapy in heart rhabdomyosarcoma treatment.

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