Downregulation of high mobility group box 1 modulates telomere homeostasis and increases the radiosensitivity of human breast cancer cells

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Abstract. The functions of the high mobility group box 1 (HMGB1) in tumor cells include replenishing telomeric DNA and maintaining cell immortality. There is a negative correlation between human telomerase reverse transcriptase (hTERT) and radiosensitivity in tumor cells. Our aim was to elucidate the relationship among HMGB1, telomere homeostasis and radiosensitivity in MCF-7 cells. In this study, we established stably transfected control (MCF-7-NC) and HMGB1 knockdown (MCF-7-shHMGB1) cell lines. The expression of HMGB1 mRNA and the relative telomere length were examined by real-time PCR. Radiosensitivity was detected by clonogenic assay. The protein expressions were determined by western blot analysis. The telomerase activity was detected by PCR-ELISA. Proliferation ability was examined by CCK-8 assay. Cell cycle and apoptosis were examined by flow cytometry. DNA damage foci were detected by immunofluorescence. ShRNA-mediated downregulation of HMGB1 expression increased the radiosensitivity of MCF-7 cells, and reduced the accumulation of hTERT and cyclin D1. Moreover, knockdown of HMGB1 in MCF-7 cells inhibited telomerase activity and cell proliferation, while increasing the extent of apoptosis. Downregulation of HMGB1 modulated telomere homeostasis by changing the level of telomere-binding proteins, such as TPP1 (PTOP), TRF1 and TRF2. This downregulation also inhibited the ATM and ATR signaling pathways. The current data demonstrate that knockdown of HMGB1 breaks telomere homeostasis, enhances radiosensitivity, and suppresses the repair of DNA damage in human breast cancer cells. These results suggested that HMGB1 might be a potential radiotherapy target in human breast cancer.

Introduction

The high mobility group box 1 (HMGB1) is an abundant and ubiquitous chromatin associated protein in mammals, which acts as a DNA chaperone in transcription, replication, recombination and repair (1). HMGB1 bends DNA and promotes the access of transcriptional protein assemblies to specific DNA targets. HMGB1 plays an important role in non-homologous end-joining, mismatch repair, and nucleotide excision repair pathways (2,3) and enhances ligation reactions of DNA double-strand breaks (DSBs) (4,5).

HMGB1 is involved in tumor development, proliferation, invasion, and metastasis, and its high levels are associated with a poor clinical prognosis. However, HMGB1 may play a conflicting dual role in tumors (6), and one study suggested that HMGB1 levels may correlate with radiosensitivity (7), although the underlying mechanism is unclear.

Telomeres are specialized DNA-protein complexes found at the ends of eukaryotic chromosomes. Telomeres are composed of a variable number of TTAGGG sequences repeated in tandem and associated proteins (8). Telomerase is detected in ~90% of all malignant tumors (9) and is a highly attractive target for cancer therapeutics. Our previous research suggested radiosensitivity of Hep-2 cells (human laryngeal squamous carcinoma cells) negatively correlated with telomere length, and positively correlated with telomerase activity (10). Thus, telomere homeostasis is closely related to radiosensitivity, and increased radiosensitivity can help control the rate of tumor growth.

A couple of studies have focused on the roles of HMGB1 in telomere biology. One study showed that HMGB1 had no effect on telomerase activity in the plant Arabidopsis thaliana, and that varying the expression of HMGB1 did not cause any obvious changes in chromatin structure (11). However, Polanska et al (12) showed that knockout of the HMGB1 gene in mouse embryonic fibroblasts (MEFs) resulted in a decline

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Abbreviations: HMGB1, high mobility group box 1; hTERT, human telomerase reverse transcriptase; hTR, human telomerase RNA; MEFs, mouse embryonic fibroblasts

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in telomerase activity and telomere dysfunction, while overexpression of HMGB1 enhanced telomerase activity. Together, these findings indicate that HMGB1 is indispensable for telomere homeostasis, but that the relationship between HMGB1 and telomere biology remains unclear in mammalian cells.

The present study was designed to determine the effect of changing the expression of HMGB1 on telomere in human cancer cells. Specifically, we investigated the role of HMGB1 on telomere homeostasis and radiosensitivity in MCF-7 human breast cancer cells.

Materials and methods

Cell lines, transfection, plasmids and reagents. MCF-7 cells were obtained from the Key Laboratory of Tumor Biological Behavior of Hubei Province and incubated under 5% CO₂ at 37°C in RPMI-1640 medium containing 10% fetal bovine serum. Transfections were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for plasmids and shRNA. The Cell Counting Kit-8 (CCK-8) was purchased from Boster (Wuhan, China).

Construction of stably transfected cell lines. For the construction of HMGB1 knockdown vectors, a human shRNA 5'-GCT CAAGGAGAATTGTGAA-3' targeted to HMGB1 was selected, the sequence had the strongest interference effect from the reference; and then a scrambled human shRNA sequence 5'-GCTCTTGGAGCAGTTCCGATATC-3', possessing limited homology to human genes, served as the negative control (13). The shRNAs were synthesized and subcloned into the pGPU6/GFP/Neo shRNA vector (GenePharma, Shanghai, China). The resultant vectors were named as pGPU6/GFP/Neo-HMGB1 and pGPU6/GFP/Neo-shNC. HMGB1 underexpressing cells and negative control cells were selected using 600 µg/ml G418 (Merck) for 5 weeks. The resultant stably transfected cell lines were named as MCF-7-shHMGB1 and MCF-7-NC, respectively.

Real-time PCR to detect the expression of HMGB1 mRNA. Total RNA was extracted from the stably transfected cells, and cDNA was synthesized using a reverse transcriptase kit (Fermentas, Canada) at 42°C for 10 min, followed by 95°C for 2 min. Duplicate PCR reactions were performed using the Takara real-time PCR kit (Takara Bio, Japan) according to the manufacturer's instructions. Samples were preincubated at 95°C for 30 sec followed by 40 cycles of 95°C for 5 sec, 60°C for 15 sec and 72°C for 30 sec. HMGB1 forward and reverse primers were 5'-ATATGGCAAAAGCGGAC AAG-3' and 5'-GCAACATCACAAATGGGACAG-3' (13). The β-actin forward and reverse primers were 5'-TGGCAACCCA GCACATTGAA-3' and 5'-CTAAGTCTATGTCGCGCCTAG AAGCA-3'. All experiments were repeated at least three times. Thermal amplification was carried out on an Mx3000P qPCR system (Stratagene, La Jolla, CA, USA), and the results were analyzed using the MXP3000 analysis program.

Clonogenic assay. Cells were plated in 6-well culture flasks. After 24 h, cells were irradiated with graded doses (0, 1, 2, 4, 6, 8 and 10 Gy) using an X-ray generator (Primus High-Energy Siemens) at a dose rate of 2 Gy/min. Cells were then cultured under 5% CO₂ at 37°C for 14 days. The colonies were fixed and stained with crystal violet (1% in absolute ethanol). Cell survival was measured by counting the colonies containing >50 cells. The data were entered into the linear-quadratic model, and the survival curve of each group was demonstrated using Graphpad Prism 5 software. Radiobiological parameters were calculated according to the survival curves.

Western blot analysis. The expression of HMGB1, hTERT, Cyclin D1, CDC25C (Abcam, Cambridge, UK), ATM, ATR, phosphor-ATM, phosphor-ATR, TRF1, TRF2, PTOP, rH2AX (Cell Signaling Technology, Danvers, MA, USA), and GAPDH (Santa Cruz Bio, Dallas, TX, USA; as a loading control) were determined by western blotting as described previously (14). All experiments were repeated three times. The results were analyzed by ImageJ software.

DNA extraction and real-time PCR to determine relative telomere length. Genomic DNA was extracted from cells by standard procedures using the TIANamp Genomic DNA kit (Tiangen Bio, Beijing, China) and stored at 4°C. Relative telomere length was determined by using qRT-PCR as described by Cawthon (15). Duplicate PCR reactions were performed using the Takara real-time PCR kit (Takara Bio, Japan) according to the manufacturer’s instructions. The telomere and single copy gene specific primers used were as follows: tel1, 5'-GTTTTTGTAGGTTAGGGTTAGGGTTAGGGTGAG GGT-3', tel2, 5'-TCCCACTTCTTCCTCCCTATCCCTA TCCCTATCCCTA-3'. 36B4u, 5'-CAGCAAGTGGGAAGG TGTAATCC-3'. 36B4d, 5'-CCATTCCTCATCACAACGGG TACA-3'. The primers were synthesized by Sangon Biotech (Shanghai, China). The cycling conditions consisted of preincubation for 5 sec at 95°C, followed by 35 cycles of 95°C for 15 sec and 54°C for 2 min. Thermal amplification was carried out using Mx3000P (Stratagene), and the results were analyzed using the MXP3000 analysis program. All experiments were repeated three times.

PCR-ELISA assay. Protein concentrations were determined by the BSA assay following cell lysis. The telomerase activity of each sample was determined using the Telo-TAGGG Telomerase PCR-ELISA kit (Roche, Basel, Switzerland). The absorbance of each sample was determined at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA) (with a blank reference wavelength of ~690 nm) 30 min after addition of the stop reagent. Data were normalized using the Renilla luciferase assay. Each experiment was performed three times in triplicate wells.

Cell proliferation assay. Cells diluted with RPMI-1640 medium containing 10% fetal bovine serum, were seeded at 10⁴ cells/well in 96-well plates and cultured in 100 µl culture medium, six identical wells were used for each sample. After 24 h, 10 µl of CCK-8 was added to each well, and the plates were incubated at 37°C for 2 h. The absorbance of each well was then read at 450 nm using a 96-well plate reader. Each experiment was performed at least three times in triplicate wells.

Analysis of cell cycle and apoptosis by flow cytometry. The cell cycle was assessed in cells without irradiation and cells
exposed to 6 Gy of ionizing radiation, and then incubated for the indicated times. Cells were fixed in 70% ethanol overnight, and then treated with RNase for 20 min before addition of 5 mg/ml propidium iodide, and analysis by flow cytometry (Beckman Coulter, Brea, CA, USA). Experiments were performed in triplicate.

Apoptosis was performed using an Annexin V-PE Apoptosis Analysis kit (Sungene Bio, Tianjin, China) according to the manufacturer's instructions. Fluorescence was measured using a flow cytometer and the data were analyzed with CellQuest software. All samples were assayed in triplicate.

**Immunofluorescence.** Cells were fixed with 4% formaldehyde for 15 min and then permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. After treatment with blocking solution, cells were incubated with the primary antibody overnight at 4˚C, washed, and incubated with the secondary antibody. Nuclei were stained with DAPI (Sigma, San Francisco, CA, USA) for 5 min at room temperature. Fluorescence was observed using a confocal microscope (Carl Zeiss LSM710, Germany).

**Statistical analysis.** All data are expressed as means ± SD. Student's t-test was used to determine statistical significance at p<0.05. SPSS17.0 and Graphpad Prism 5 software were used for the statistical analyses.

**Results**

**Downregulation of HMGB1 increases the radiosensitivity of MCF-7 cells.** The effect of shRNA-HMGB1 on the expression of HMGB1 was determined in MCF-7 cells (Fig. 1). HMGB1 expression was not affected in MCF-7-NC cells compared with the parental MCF-7 cells. However, there was a significant inhibition of HMGB1 expression in MCF-7 cells stably expressing shRNA-HMGB1.

Control and shRNA-HMGB1 transfected MCF-7 cells were exposed to different doses of radiation. After cell clones were counted, the survival curves were plotted to evaluate the radiobiological parameters of each group. Compared to the negative MCF-7 cell control group, the survival fractions of the shRNA-HMGB1 group were much lower at each dose of radiation (Fig. 2). Plating efficiency and survival fraction

**Figure 1.** Real-time RT-PCR determination of HMGB1 mRNA. HMGB1 expression was not affected in MCF-7-NC cells compared with the parental MCF-7 cells, whereas significant inhibition of HMGB1 expression was found in stable HMGB1 silenced MCF-7 cells versus control. ***p<0.001.

**Figure 2.** The cell survival curve of MCF-7-NC and MCF-7-shHMGB1 cells. Cells were irradiated with 0, 1, 2, 4, 6, 8 and 10 Gy and then plated for colony formation for 2 weeks. Surviving fraction of cells after irradiation in 2 Gy (SF2) was 0.7756±0.0016 and 0.5732±0.0031 (p<0.01), suggesting that HMGB1 downregulation induced radiosensitive of MCF-7 cells.

**Figure 3.** Protein levels of (HMGB1, hTERT, TPP1, TRF1, TRF2, cyclin D1, ATM, ATR, phospho-ATR, phospho-ATM, rH2AX and GAPDH) were examined by western blotting. (A) The effect of downregulating HMGB1 on telomere homeostasis. (B) The effect of downregulating HMGB1 on the repair of double-strand breaks and cell cycle proteins. (C) The effect of downregulating HMGB1 on the expression of CDC25C, TRF2, phospho-ATR and rH2AX after irradiation.
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was calculated. Surviving fraction of cells after irradiation in 2 Gy (SF₂) was 0.7756±0.0016 and 0.5732±0.0031 (p<0.01), suggesting that HMGB1 downregulation induced radiosensitivity of MCF-7 cells.

Downregulation of HMGB1 leads to telomere dysfunction, inhibits DNA damage repair and modulates the cell cycle. To evaluate whether the level of HMGB1 correlates with telomere homeostasis and the repair of DSBs in MCF-7 cells, we constructed cell lines with stable downregulation of HMGB1. Fig. 3A indicates that the expressions of hTERT, PTOP, TRF1 and TRF2 were attenuated by downregulating HMGB1. These data suggest that telomere homeostasis was disrupted.

The expression of ataxia telangiectasia mutated (ATM), ataxia telangiectasia rad3-related (ATR), and cyclin D1 were reduced, while the expression of phosphor-ATM, phosphor-ATR, rH2Ax and CDC25C were increased in MCF-7-shHMGB1 cells compared to MCF-7-NC cells (Fig. 3B). When exposed to radiation, rH2Ax, phosphor-ATR and CDC25C were further increased when HMGB1 was downregulated. The increase in rH2AX and phosphor-ATR protein levels was greater after 4 Gy exposure than after 2 Gy radiation exposure. However, TRF2 decreased when HMGB1 was downregulated after irradiation (Fig. 3C).

**Downregulation of HMGB1 decreases telomere length and telomerase activity.** To investigate the role of HMGB1 in modulating telomere length, MCF-7-shHMGB1 and MCF-7-NC cells were cultured for 15 population doublings, and telomere length was measured by real-time PCR. The average telomere length of MCF-7-shHMGB1 cells was shorter than that in control cells (Fig. 4A). These results suggest that HMGB1 is important in maintaining telomere length in MCF-7 cells.

Telomerase activity is regarded as the primary determinant of tumor cell radiosensitivity. The telomerase PCR-ELISA technique was used to determine the effect of HMGB1 on telomerase activity. The activity of telomerase in MCF-7-shHMGB1 cells was lower than that measured in MCF-7-NC cells (p=0.012) (Fig. 4B).

**HMGB1 is involved in controlling the proliferation and cell cycle of MCF-7 cells.** Downregulation of HMGB1 inhibited the proliferation of MCF-7 cells (Fig. 5A). Exposing MCF-7-shHMGB1 cells to 2 Gy of radiation further decreased the
proliferation of these cells compared to MCF-7-NC cells (Fig. 5B) (p<0.05).

**Downregulating HMGB1 decreases the proportion of MCF-7 cells in the S phase.** When exposed to radiation, there was an increase in the percentage of MCF-7-shHMGB1 cells in the G2/M phase compared to MCF-7-NC cells (p=0.0305). There were no significant differences in the number cells in the G1 phase between MCF-7-NC and MCF-7-shHMGB1 cells (Fig. 6). Downregulation of HMGB1 promotes apoptosis of MCF-7 cells. MCF-7-NC and MCF-7-shHMGB1 cells were irradiated with 6 Gy and incubated for 24 h. The percentage of cells undergoing apoptosis was measured by flow cytometry. Downregulating HMGB1 enhanced apoptosis in irradiated MCF-7 cells (p=0.003) (Fig. 7).

**Decreasing the expression of HMGB1 inhibits the repair kinetics of DNA damage induced by ionizing radiation.** An immunofluorescence assay was used to establish whether decreasing the expression of HMGB1 affected the repair
Figure 8. The downregulation of HMGB1 attenuates the repair of DNA damage induced by ionizing irradiation. The spontaneous foci in MCF-7-NC and MCF-7-shHMGB1 cells were detected. The cells were then exposed to 4 Gy and incubated for 1 h. Results from three representative images for damaged foci are shown.

Figure 7. Enhanced ionizing radiation induced apoptosis by downregulating HMGB1. MCF-7-NC and MCF-7-shHMGB1 cells were irradiated with 6 Gy and incubated for 24 h. The percentage of apoptotic cells was measured by flow cytometry. (A) Representative results of different groups are shown. **p<0.01. (B) Data are presented as means ± SD from three independent experiments.
kinetics of DNA damage. There were significantly more foci indicating spontaneous damage in the MCF-7-shHMGB1 cells compared to the control cells (p<0.05). When exposed to 4 Gy of ionizing radiation and stained 1 h later to identify the damaged foci, HMGB1 underexpression was found to attenuate the ability of MCF-7 cells to repair these sites (Fig. 8).

**Discussion**

The results of the present study show that decreasing the expression of HMGB1 was associated with damage to telomeres and increased radiosensitivity of breast cancer cells. This suggests that HMGB1 plays crucial roles in the regulation of telomeres and the response of cells to DNA damage.

One previous study suggested that increasing the expression of HMGB1 suppressed cell growth by initiating G1 arrest and apoptosis in MCF-7 cells, furthermore showing that HMGB1 suppressed the growth of MCF-7 tumor xenografts in nude mice, suggesting that HMGB1 functions as a tumor suppressor and radio-sensitizer in breast cancer cells (16).

Previous research indicated that radiosensitivity negatively correlates with telomere length (17). In our study, the expression of HMGB1 was correlated with telomere length and radiosensitivity in breast cancer cells. Furthermore, we found that downregulating HMGB1 enhanced the radiosensitivity of these cells, decreasing both telomere length and telomerase activity.

Lower telomerase activity correlated with higher radiosensitivity, and could lead to a decrease in telomere length. Both the hTERT and hTR subunits are required for telomerase activity. In our research, we found that the downregulation of HMGB1 decreased the level of hTERT, an effect that may explain the decrease in telomerase activity. In contrast, another report indicated that there was no change in RNA and protein level of TERT in HMGB1 knockout MEFs (12). The difference between these results may be due to differences in the cell lines that were studied. Additional research in other cell lines is required to determine the mechanism by which HMGB1 influences the telomere homeostasis.

The main role of cyclin D1 is to regulate the shift from the G1 to the S phase of the cell cycle. The accumulation of hTERT and cyclin D1 was able to attenuate the radiosensitivity of MCF-7 cells (18), and there was a direct positive correlation between the levels of cyclin D1 expression and resistance to radiation in tumor cells (19). We found that decreasing the expression of HMGB1 led to a significantly decrease in cyclin D1. Thus, the enhanced radiosensitivity seen in cells with diminished HMGB1 may occur through the modulation of cyclin D1 expression. Furthermore, because a decrease in cyclin D1 can inhibit the G1/S transition, this may explain the significant inhibition of proliferation that was seen in MCF-7-shHMGB1 cells. In addition, the increased levels of CDC25C protein that were seen could promote the G2 to M phase transition. Because the M phase is the most radiosensitive phase of the cell cycle, this may also have enhanced the radiosensitivity of these cells.

ATM and ATR protein kinases are major upstream checkpoint kinases for the DNA damage response, at the G1/S transition, ATR promotes progression and prevents stasis (20). In our research, downregulation of HMGB1 inhibited the expression of ATM and ATR in MCF-7 cells. Other studies have demonstrated that the inhibition of ATM or ATR results in increased radiosensitivity (21,22), and that the activation of ATR kinase during the G1 phase facilitates the repair of ionizing radiation-induced DNA damage (23).

Our results showed that the downregulation of HMGB1 enhanced the expression of phosphor-ATM and phosphor-ATR which could manifest the level of DSB damage accumulation. The protein level of γH2AX (marker of DSBs) also increased significantly when HMGB1 was downregulated. Furthermore, when exposed to ionizing radiation, the levels of phosphor-ATR and rh2AX increased significantly in HMGB1 knockdown cells, and the protein levels of phosphor-ATR and rh2AX were more enhanced when exposed to 4 Gy than to 2 Gy of radiation.

Many studies have shown that the telomere serves as a target in cancer treatment, especially in radiotherapy. The stability of telomeres is maintained by telomerase as well as associated proteins. Previous research suggested that high levels of TPP1 and POT1 are directly associated with poor radiosensitivity in LSCC cells, while low levels of TPP1 and POT1 have the opposite effect (14). In addition, suppression of TPP1 expression resulted in telomere dysfunction and enhanced radiation sensitivity in telomerase-negative osteosarcoma cell line (24). TPP1 helped to stabilize the TRF1-TIN2-TRF2 interaction and promoted the formation of the six-protein complex. Overexpression of TPP1 enhanced the association between TIN2 and TRF2, while decreasing the expression of TPP1 reduced the ability of endogenous TRF1 to associate with the TRF2 complex (25).

Tumors with a high abundance of the TRF1 protein exhibited greater telomerase activity and longer telomeres than tumors with lower TRF1 protein levels. This indicated that telomere length was significantly associated with TRF1 protein levels (26). The present study showed that TPP1 and TRF1 were downregulated when HMGB1 expression was decreased. This was accompanied by an increased radiosensitivity in breast cancer cells, suggesting the involvement of TPP1 and TRF1. Another study suggested that TPP1 and TRF1 helped enhance the radio resistance of breast cancer cells. Specifically, the expression of TPP1 and TRF1 was significantly increased in radio resistant cell lines than the parent cell lines, and in addition, after silencing the TPP1 gene, the radio resistant cell lines significantly decreased their radio resistance and telomerase activities (27).

TRF2 is recruited to sites of DNA damage and plays a critical role in the DNA damage response (28). Radiosensitization in U2OS cells may be related to shortening of the telomeres and decreases in TRF2 (29). Upon removal of TRF2 from TRF2<sup>p53</sup> p53<sup>−/−</sup> MEFs, damage to telomeres was observed at most chromosome ends, the telomeres lose the 3' overhang and are processed by the non-homologous end-joining pathway (30).

In our study, TRF2 also decreased after HMGB1 knockdown in MCF-7 cells, and the same results occurred when these cells were irradiated. Thus, TRF2 may play an important role in the radiosensitivity of MCF-7 cells.

Previous research investigating the effect of HMGB1 on telomere-binding proteins has been limited. In our study, there was an obvious correlation between the expression of...
HMGB1 and telomere dysfunction, which could explain the observed enhanced radiosensitivity. HMGB1 protects cells against apoptosis by influencing the stability of telomeres (31). The results from our study showed that downregulation of HMGB1 increased apoptosis in MCF-7 cells exposed to ionizing radiation. This might have resulted from the downregulation of HMGB1, leading to telomeres being shortened to a critical length, thereby initiating apoptosis. The increased apoptosis may in turn be involved in the increased radiosensitivity.

The results of this study demonstrate that the decreasing HMGB1 levels promote telomere dysfunction and DNA damage, and confer radiosensitivity in human breast cancer cells. In addition, we provide evidence of correlation among HMGB1 expression, telomere homeostasis and intrinsic radiosensitivity, suggesting that HMGB1 is a potential target in the radiotherapy of breast cancer.

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