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Potentiality of phosphorylation of BRCA1 at Ser 1524 to activate p21 in response to X-ray irradiation

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Abstract The breast and ovarian cancer susceptibility gene BRCA1 encodes a nuclear phosphoprotein, which functions as a tumor suppressor gene. Many studies suggested that multiple functions of BRCA1 may contribute to its tumor suppressor activity, including roles in cell cycle checkpoints, apoptosis and transcription. It is postulated that phosphorylation of BRCA1 is an important means by which its cellular functions are regulated. In this study, we employed phospho-Ser-specific antibody recognizing Ser-1524 to study BRCA1 phosphorylation under conditions of DNA damage and the effects of phosphorylation on BRCA1 functions. The results showed that 10 Gy X-ray treatment significantly induced phosphorylation of Ser-1524 but not total BRCA1 protein levels. The expression both of p53 and p21 increased after irradiation, but ionizing radiation (IR) -induced activation of p21 was prior to that of p53. The percentages of G0/G1 phase remarkably increased after IR. In addition, no detectable levels of 89 kDa fragment of PARP, a marker of apoptotic cells, were observed. Data implied that IR-induced phosphorylation of BRCA1 at Ser-1524 might activate p21 protein, by which BRCA1 regulated cell cycle , but play no role in apoptosis.

Key words BRCA1, Phosphorylation, Cell cycle, Apoptosis

CLC number Q6

1 Introduction

BRCA1 is a tumor suppressor gene that is mutated in a high percentage of hereditary breast and ovarian cancers [1]. The human *brca1* gene has been mapped to chromosome 17q21 and encodes a protein of 1863 amino acid residues. The *brca1* gene is conserved in mammals, but not in lower animals. Analysis of the BRCA1 protein has revealed that it contains an N-terminal Ring-finger domain and two C-terminal BRCT (BRCA1 -C-Terminal) domains, both involved in protein-protein interactions. Many studies suggested that BRCA1 might function as a scaffold in the assembly of a multi-protein complex, which plays a role in gene transcription, DNA damage repair, and transcription- coupled DNA damage repair [2-5].

Many studies implicate that BRCA1 may play a role in controlling gene transcription. For example, BRCA1 can bind to p53 and enhance its transcription activity at the promoter of p21 [6,7]. A recent study showed that BRCA1 could selectively coactivate the p53 transcription factor towards genes that direct DNA repair and cell cycle arrest but not towards those that

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direct apoptosis [8]. In response to DNA damage, the BRCA1 protein become rapidly hyperphosphorylated at multiple sites by several kinases including ATM and Chk2 (hCds1) [9,10]. Moreover, there is a qualitative difference in the phosphorylation states of BRCA1 between ionizing radiation (IR) and UV radiation. For instance, BRCA1 Ser-1423 and Ser-1524 are rapid phosphorylated after IR but not after UV [11]. It has been found that prior ATM/ATR-dependent phosphorylation of BRCA1 at Ser-1423 or Ser-1524 regulates the ability of ATM/ATR to phosphorylate $p53^{Ser\text{-}15}$ efficiently [12]. Phosphorylation of $p53^{Ser\text{-}15}$ is necessary for an IR-induced G_1/S arrest via transcriptional induction of the cyclin-dependent kinase inhibitor p21.

It is likely that phosphorylation of specific residues determines the role of BRCA1 in damaged cells. Thus, in this paper we investigated BRCA1 phosphorylation under conditions of DNA damage and the effects of phosphorylation on BRCA1 functions.

2 Materials and methods

2.1 Cell culture and irradiation

The human breast cancer cell line MCF-7 was maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) with 10% fetal bovine serum in 5% CO_2 at 37°C. To induce DNA damage, cells were irradiated to 10Gy using an X-ray irradiator.

2.2 Western blotting analysis

At 0, 0.5, 1, 2, 4, 6, 8 and 12 h post-irradiation, cells were washed twice with cold phosphate buffered solution (PBS) and lysed in RIPA buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate and 0.05% sodium dodecyl sulfate). Protein concentrations were determined with the BCA assay. The 20 μg of total cellular protein from the samples were boiled for 5 min, subjected to 10% or 15% SDS-PAGE, and transferred to a Hybond membrane (Amersham). The membrane was blocked in TBS containing 3% BSA and 0.1% Tween 20 and probed with a primary antibody and secondary antibody. The primary antibodies used were rabbit polyclonal antibody to BRCA1 (Cell Signaling Technology, Inc.), BRCA1 phospho-Ser1524 (Cell Signaling Technology, Inc.), p53 (Cell Signaling Technology, Inc.), p21 (Cell Signaling Technology, Inc.), PARP (a poly ADP-ribose polymerase) (Santa Cruz Biotechnology, Inc.) and β-actin (Santa Cruz Biotechnology, Inc.). The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG (Millipore Corporation). Secondary probes were detected by ECL Western blotting detection reagents and analysis system (Amersham).

2.3 Cell cycle distribution analysis

Following incubation, the cells were trypsinized, washed twice with cold PBS, fixed with 70% ethanol in PBS for 30 min, and then stained with a solution containing 5μg/mL propidium iodide (PI), 10 kU/mL RNase (Sigma), and 0.005% Triton-100 in the dark for 30 min at room temperature before flow cytometric analysis. The samples were detected with FASC Calibur (Becton, Dickinson and Company, USA). A minimum of 10 000 cells analyzed in each sample served to determine the percentages of cells in each phase of the cell cycle using WinDMI 2.9 and Cylchred 1.0 softwares. Three independent experiments were tested.

2.4 Statistical analysis

The means of three independent experiments (triplicate samples) ±SD were shown, and statistical calculation was done using Microsoft Excel. For statistical significance, Student's *t*-test values were calculated. Significance was defined as a minimum of $p<0.05$ for each comparison.

3 Results

3.1 Radiation-induced phosphorylation of BRCA1 at Ser-1524

SDS extracts prepared from the irradiated cells were examined for BRCA1 activation and phosphorylation by Western blotting using anti-BRCA1 antibody and anti-phosphoserine-1524 peptide antibody. We found no remarkable change of total BRCA1 protein levels in response to irradiation (Fig.1). In contrast, phosphorylation of BRCA1 Ser-1524 was induced significantly. Phosphorylation was detected as early as 0.5 h post-irradiation and increased in a

time-dependent manner.

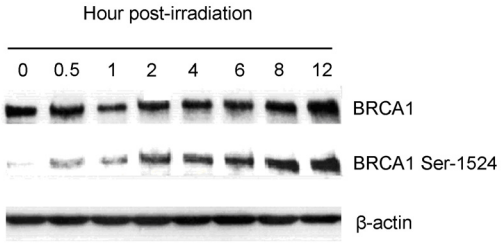

Fig.1 The levels of total BRCA1 protein and phosphorylation of BRCA1 Ser-1524 after IR. Western blot analysis was carried out using anti-BRCA1 antibody, anti-phosphoserine 1524 BRCA1 peptide antibody, and anti-β-actin antibody as normalizing control.

3.2 Role of BRCA1 phosphorylation in p21 activation

p21 is a well-characterized BRCA1 target gene[13]. We investigated the expression of p21 by Western blotting, and found that p21 levels increased as early as 1 h and lasted 12 h post-irradiation.

As is well known, p53 is an upstream activator of p21. At the same time, many studies demonstrate that BRCA1 can bind to p53 and coactivate transcription activity of p21 [6,7]. So we investigated the expression of p53 and found p53 levels were induced at 6 h post-irradiation (Fig.2).

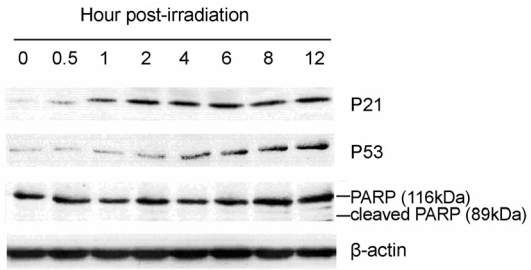

Fig.2 The expression of P21, P53 and PARP after IR.

3.3 Analysis of IR-induced cell-cycle arrest or apoptosis after irradiation

To investigate the role of BRCA1 phosphorylation in cell growth, we evaluated cell-cycle distribution after irradiation by flow cytometry (Table 1). At 1 h after irradiation, cells showed slight accumulation in the G0/G1 phase, but at 4h post-irradiation the increased level of G0/G1 cells became obvious. The percentage of G0/G1 cells was elevated at 54.03% ~ 57.00%, significantly greater than the control (p<0.05). The G0/G1 phase population decreased slightly at 6 h post-irradiation but significantly increased at 12 h post-irradiation (p<0.01). Moreover, S phase population significantly decreased after irradiation compared with the control cells (p<0.05).

Table 1 Changes (in %) in cell cycle distribution of MCF-7 cells at different hours post-irradiation

Time / h	G0/G1	S	G2/M
0	54.03±0.56	32.58±1.14	13.39±1.61
1	54.43±1.39	27.27±1.56(a)	18.30±2.33
4	57.00±1.10(a)	28.32±1.43(a)	14.68±0.70
6	56.72±1.75	25.23±1.57(a)	18.05±3.02
12	58.94±0.99(b)	28.14±1.59(a)	12.93±2.42

*Data represent mean± SEM，n=3. The differences among data of individual groups were tested with the analysis of Student's t-test. (a) p<0.05, (b) p<0.01 vs. control.

We also investigated the level of total PARP protein. The 89 kDa fragment of PARP, which accompanied with apoptosis, was detected by Western blotting. The total PARP levels increased after irradiation, but levels of p89 were not detectable (Fig.2*)*.

4 Discussion

An accumulating body of evidence indicates that BRCA1 may play multiple and important roles in damage cells, including roles in cell cycle checkpoints, apoptosis, transcription and DNA repair [2-5]. BRCA1 has been found in a large nuclear protein complex, named BRCA1-associated surveillance complex, which was believed to be an important sensor to monitor the genome for damage and to signal to downstream proteins [14]. One mechanism that may explain the function of BRCA1 is through phosphorylation. In response to DNA damage, the BRCA1 protein become rapidly hyperphosphorylated at multiple sites by several kinases, such as ATM, ATR and Chk2 [9,11,15-17]. Recent study suggested the phosphorylation levels of BRCA1 might determine the fate of cells in the decision between cell cycle arrest and apoptosis [18]. Serine 1524 in BRCA1, a major phosphorylation site by ATM/ATR, was found localized to nuclear foci within 2 h after DNA damage, suggesting that phosphorylation might regulate the localization of BRCA1[19]. Moreover, prior ATM/ATR-dependent phosphorylation of BRCA1 at

Ser-1524 regulated the ability of ATM/ATR to phosphorylate $p53^{Ser-15}$ efficiently [12], while the latter is necessary for transcriptional induction of the cyclin-dependent kinase inhibitor p21. It is tempting to speculate that the phosphorylation of BRCA1 at Ser-1524 might involve in modulating p21 expression and cell cycle arrest following DNA damage.

In this study, we observed that phosphorylation of BRCA1 at Ser-1524 but not total BRCA1 protein level was significantly induced by 10Gy X-irradiation in MCF-7 cells, which suggested that phosphorylation rather than total protein levels might play more important role in BRCA1 function. Similar results were obtained in 4Gy X-irradiation (data not shown). It is not clear how the functions of BRCA1 are modulated by phosphorylation. Perhaps the phosphorylation states of BRCA1 determine its ability to bind to other proteins and affect its biochemical activities in DNA damage repair or gene transcription [8], or may affect its cellular localization or stability.

p21 is a well-characterized BRCA1 target gene[13]. Thus we investigated the effect of BRCA1 phosphorylation on transactivation of p21. Results showed that p21 levels increased as early as 1h post-irradiation and this increase in p21 expression remained until 12h, which was corresponding to the phosphorylation of BRCA1 at Ser-1524. Similar results were found in 4Gy X-irradiation (data not shown). p53 is a well-known upstream activator of p21, many studies demonstrated that BRCA1 can interact with p53 and enhance its transcription activity at the promoter of p21, thus the expression of p53 was also examined. The level of p53 was found increased at 6h post-irradiation. Here, we noted that IR-induced activation of p21 was prior to that of p53, which implicated that phosphorylation of BRCA1 at Ser-1524 might induce p21 activation directly or through other signal pathway except for p53 pathway. Previous study reported that BRCA1 could transactivate expression of p21 in a p53-independent manner [13]. However, further, more detailed studies are required to verify this hypothesis.

Moreover, we measured cell cycle distribution and observed a significant increase in G0/G1 phase and a remarkable decrease in S phase after 10 Gy X-irradiation. Previous study reported that in normal growing cells, BRCA1 is phosphorylated in a cell cycle-dependent manner, and the protein undergoes hyperphosphorylation in S phase and is dephosphorylated after M phase [20,21]. It has been also shown that DNA damage induced both nuclear redistribution of BRCA1 and an increased phosphorylation of the protein through DNA damage-activated kinases such as ATM, ATR, and Chk2. In this study, we observed a significant decrease in number of S phase cells but dramatic increase in phosphorylation of BRCA1 at Ser-1524, which suggested that this phosphorylation was induced by ATM in response to DNA damage rather than a cell cycle-dependent manner. These results reinforce a model wherein phosphorylation of specific residues of BRCA1 after DNA damage affects its localization and function. In addition, no detectable levels of p85 (PARP cleavage fragment), a marker of apoptotic cells, were found in MCF-7 after irradiation. These data implied that BRCA1 phosphorylation may contribute to direct damage cells towards cell cycle arrest but not apoptosis.

Here our results promote the possibility that phosphorylation of specific residues may affect the role of BRCA1 in response to IR. Phosphorylation of BRCA1 at Ser-1524 might potentially activate p21 protein, by which BRCA1 regulates cell cycle.

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