

α B-crystallin enhances radioresistance in basal-like breast cancer cells by suppressing caspase-3 activation

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Abstract

Objective: For most patients with breast cancer undergoing breast-conserving surgery, radiotherapy reduces local recurrence and mortality. However, in basal-like breast cancer, radiotherapy is less effective due to the cells' intrinsic radioresistance. Understanding the molecular mechanism of radioresistance in basal-like breast cancer is crucial for improving treatment and outcomes.

Methods: α B-crystallin levels were determined in different breast cancer cell lines, including basal-like breast cancer cell lines (HCC1599, MDA-MB-468, MDA-MB-231) and luminal breast cancer cell lines (MDA-MB-453, BT-474, MCF-7). To further investigate the role of α B-crystallin in breast cancer cells, we utilized short hairpin RNA lentivirus particles to knock down α B-crystallin expression in MDA-MB-231 cells and overexpressed it in MCF-7 cells via lentivirus-mediated delivery of α B-crystallin. Cell migration and invasion were assessed using transwell assays, and radiosensitivity was evaluated via colony formation assays. Flow cytometry was employed to quantify

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apoptotic cells and western blot was performed to explore the expression levels of invasion and migration-related proteins and apoptotic proteins. The levels of γ -H2AX and the co-localization of α B-crystallin with Caspase-3 in cells were evaluated using immunofluorescence microscopy. Immunoprecipitation followed by western blot was used to detect the interaction of α B-crystallin with Caspase-3.

Results: We have identified α B-crystallin as a pivotal regulator of migration and radiosensitivity in basal-like breast cancer. Repression of α B-crystallin expression enhanced radiation-induced DNA damage and apoptosis in basal-like breast cancer cells. Moreover, knockdown of α B-crystallin diminished its interaction with Caspase-3, resulting in an amplified activation of Caspase-3 triggered by irradiation, intensified apoptosis, and heightened radiosensitivity in basal-like breast cancer cells.

Conclusions: The overexpression of α B-crystallin not only enhances the migratory capacity of basal-like breast cancer cells but also confers radioresistance to the cells by directly interacting with Caspase-3 and inhibiting its activity. Suppression of α B-crystallin enhances radiation-induced cell death, thereby improving the radiosensitivity of basal-like breast cancer cells.

Keywords

Basal-like breast cancer, α B-crystallin, Caspase-3, apoptosis, radioresistance

Introduction

Breast cancer is the most prevalent malignant neoplasm among women globally and poses a significant threat to female health.¹ According to the molecular characteristics, breast cancer can be classified into four subtypes: luminal epithelial type A, luminal epithelial type B, Her2 (human epidermal growth factor receptor 2) overexpression type, and basal-like type. The basal-like subtype of breast cancer, comprising approximately 15% to 20% of all breast cancers, is characterized by its highly aggressive nature, frequent recurrence, absence of targeted therapeutic options, and unfavorable prognosis.² Breast cancer is primarily managed through surgical intervention, radiotherapy, or a combination thereof, alongside systemic chemotherapy, endocrine therapy, biological therapy, or a combination of these modalities.³ The administration of radiotherapy is considered a crucial component in the treatment regimen for the majority of patients with breast cancer who undergo breast-conserving surgery, as it confers substantial clinical advantages including a reduction in local tumor recurrence risk and improved overall survival rates from breast cancer.⁴ However, local tumor control rates remain suboptimal for basal-like breast cancer. In the case of patients with this particular subtype, inherent radioresistance of cancer cells constitutes a primary obstacle to the efficacy of radiotherapy. Therefore, elucidating the molecular mechanism underlying radioresistance in basal-like breast cancer holds significant potential for enhancing the clinical efficacy of treatment strategies for this subtype in future therapeutic interventions.

The gene test data demonstrate that α B-crystallin exhibits a significantly elevated expression level in basal-like breast cancer tissues, thereby facilitating cancer cell migration and invasion as well as promoting angiogenesis.^{5,6} The transplantation of tumors with

α B-crystallin overexpression in nude mice has been demonstrated to induce basal-like tumor characteristics, as evidenced by previous studies.⁷ The collective findings suggest that α B-crystallin serves as a reliable biomarker for basal-like breast cancer, a subtype with distinct molecular characteristics. Notably, studies have consistently reported α B-crystallin expression in 45% to 56% of basal-like breast cancer tissues.^{8,9} The expression sensitivity of α B-crystallin in basal-like breast cancer was found to be higher compared to CK5/6, CK14, and p63. Additionally, its specificity was observed to be higher than that of epidermal growth factor receptor and c-Kit. Therefore, α B-crystallin can be considered as a sensitive and specific marker for identifying basal-like breast cancer.^{10,11}

α B-crystallin, a member of the mammalian small heat shock protein (sHSP) superfamily, serves as a cellular chaperone that is upregulated in response to various stimuli. This upregulation effectively mitigates the accumulation of misfolded proteins and enhances cell survival.¹² The heterotopic expression of α B-crystallin in various cell types confers protection against a diverse range of apoptotic stimuli, including tumor necrosis factor- α , chemotherapeutic agents, growth factor deficiency, and oxidative stress. This protective effect is achieved through the inhibition of Caspase-3 activation and the binding to apoptotic Bcl-2 family members (Bax, Bcl-x), thereby safeguarding mitochondria and suppressing apoptosis.^{13,14} Therefore, α B-crystallin is likely to enhance radiation resistance in basal-like breast cancer by suppressing apoptosis. In this study, we observed a significant upregulation of α B-crystallin expression in basal-like breast cancer cells, which was found to facilitate cell migration and confer augmented radioresistance. Conversely, the downregulation of α B-crystallin levels demonstrated an augmented sensitivity toward radiation treatment in basal-like breast cancer, thereby potentially enhancing the efficacy of clinical radiotherapy for this particular subtype.

Materials and methods

Cell culture and irradiation

The cell lines utilized in this investigation were acquired from the Cell Bank of Type Culture Collection at the Chinese Academy of Sciences (Shanghai, China). HCC1599 (RRID: CVCL_1256), MDA-MB-468 (RRID: CVCL_0419), and MDA-MB-231 (RRID: CVCL_0062) cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco, Australia), along with 100 U/mL penicillin and 100 μ g/mL streptomycin. MDA-MB-453 (RRID: CVCL_0418), BT-474 (RRID: CVCL_0179), and MCF-7 (RRID: CVCL_0031) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) enriched with 10% FBS, 100 μ g/mL streptomycin, and 100 U/mL penicillin. The cells were irradiated using an RS2000 X-ray device (RS-2000 Pro; Rad Source, USA) at a dose rate of 1.16 Gy/min.

Transwell assay

Cell migration and invasion were assessed using Boyden chambers with a diameter of 6.5 mm and pores measuring 8 μ m (Cat. No: 353097; Corning, USA). The upper

chambers were either coated or uncoated with Matrigel (Cat. No: 356234; Corning) to detect invasion or migration, respectively. Following a 6-hour serum-free starvation period, 40,000 cells per well were seeded into the upper chambers and cultured in serum-free medium. In the lower chamber, 600 μ L of medium containing 10% FBS was added. Cells were cultured continuously for either 12 (migration) or 24 hours (invasion). Subsequently, nonmigrating cells and Matrigel in the upper chamber were eliminated using a cotton swab. The filters containing invaded cells were individually fixed using 4% paraformaldehyde, stained with 0.1% crystal violet solution, and visualized under a light microscope (IX73; Olympus, Japan).

Colony formation assay

The cell radiosensitivity was assessed using a colony formation assay. Varying numbers of cells were seeded into six-well plates (150, 200, 400, 1000, and 2000 per well). After a 24-hour incubation, the cells were exposed to X-ray irradiation at room temperature with doses of 0, 2, 4, 6, and 8 Gy. The colonies were allowed to grow for a duration of 2 weeks after radiation exposure. Subsequently, the colonies were rinsed twice with PBS solution and fixed using 70% ethanol before being stained with crystal violet solution (0.1%). Colonies consisting of more than 50 cells were counted. The percentage of colony formation rate for each group was calculated as follows: (number of clones divided by the initial number of seeded cells) multiplied by one hundred percent (%). The survival fraction (SF) for the control group was considered as one hundred percent (%), while the SF for each experimental group was determined by dividing its clone formation rate by that of the control group and multiplying it by one hundred percent (%).

Real-time quantitative PCR

Total RNA was extracted using Trizol reagent (Cat. No: 15596026CN; Invitrogen, USA). Subsequently, the extracted RNA was reverse-transcribed into cDNA using the PrimeScript RT Reagent Kit (Cat. No: RR037A; Takara, Japan). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using PowerUp™ SYBR® Green Master Mix (Cat. No: A25918; Thermo Fisher Scientific, USA) on a Life Technologies system (ViiA7; Thermo Fisher Scientific, USA). The primers for α B-crystallin were designed as follows: forward primer: 5'-GCACTTCTCCCCAGAGGAAC-3'; reverse primer: 5'-CCATTACAGTGAGGACCCC-3'. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the primers were as follows: forward primer: 5'-GCACCGTCAAGGCTGAGAAC-3'; reverse primer: 5'-TGGTGAAGACGCCAGTGG-3'. GAPDH was used as an internal normalization control. The mean values of six independent replicates for each gene were calculated. Relative gene expression levels were quantified using the $2^{-\Delta\Delta C_t}$ method.

Gene silencing and overexpression

Short hairpin RNA (shRNA) sequences targeting α B-crystallin were annealed and ligated into the pU6-shRNA-CMV-Puro lentiviral vector. The sequences of α B-crystallin

shRNA: sense, 5'-CCGGGACTAGTGCTGAAGCTTATTATTCAAGAGATAATAAGCTTCAGCACTAGTCTTTTTTG-3'; antisense, 5'-AATTCAAAAAGACTAGTGCTGAAGCTTATTATCTCTT GAATAATAAGCTTCAGCACTAGTC-3'; and target, CTAGTGCTGAAGCTTATTA. The empty vector was used as a control. Lentiviral vector pCDH-EF1-MCS-T2A-Puro carrying the cDNA of α B-crystallin was constructed for the purpose of overexpressing α B-crystallin. An empty vector was used as a negative control. The titer of all the aforementioned lentiviral vectors was 1×10^8 TU/mL. The viral stock solution was subsequently diluted at a ratio of 1:10 for the infection of target cells. All lentiviral vectors carried the puromycin resistance gene and were purchased from Sangon Biotech (Shanghai, China). Human breast cancer cell lines were infected with lentiviruses for 24 hours and then cultured in fresh medium. After incubation for 48 hours, media containing puromycin at a concentration of 1.5 μ g/mL was added to select cells that exhibited stable expression of the desired genes.

Western blot assay

Radioimmunoprecipitation assay buffer was utilized for the extraction of total proteins, and protein concentration was determined using a DC Protein Assay Kit I (Cat. No:500-0111; Bio-Rad, USA). All protein samples were subjected to denaturation at 100°C for 5 minutes. Equal quantities of protein were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis and subsequently transferred onto a PVDF (polyvinylidene fluoride) membrane. The membranes were immersed in nonfat milk (5%) to block heterogeneous antigens for 1 hour at room temperature, followed by overnight incubation with primary antibodies at 4°C. After three washes with PBST (PBS supplemented with Tween-20), the membranes were exposed to horseradish peroxidase-conjugated secondary antibody at room temperature for 1 hour. Signals were detected using an ECL (enhanced chemiluminescence) kit (Cat. No: 6883; Cell Signaling Technology, USA) and recorded utilizing a polychromatic fluorescence chemiluminescence imaging analysis system (FluorChem E; Protein Simple, USA). Relative densitometry values for the protein blots were analyzed using Image J 1.8.0 software. The details of the antibodies utilized in the western blot analysis are presented in Table 1.

Assessment of apoptosis and Caspase-3 activity

The cells were harvested 48 hours after exposure to a radiation dose of 4 Gy of X-rays. Subsequently, flow cytometry (Verse; BD Biosciences, USA) was employed to quantify apoptotic cells using the Annexin V-PE/7-AAD Apoptosis Detection Kit (Cat. No: 40295S; Cell Signaling Technology, USA), following the manufacturer's guidelines. Forty-eight hours after radiation exposure, the cells were harvested and lysed on ice for 5 minutes using a lysis buffer (Cat. No: 7018S; Cell Signaling Technology, USA). The lysates were then centrifuged at $10,000 \times g$ for 10 minutes at 4°C. The resulting supernatant was transferred to a 96-well plate at a volume of 100 μ L per well. Caspase-3 activity was subsequently measured using Caspase-3 activity assay kits

Table 1. Primary and secondary antibodies used for western blot.

Antibody	Company	Cat. No:	Clone number	Dilution
α B-crystallin	Abcam	230722	6D11	1:500
N-cadherin,	Cell Signaling Technology	13116	D4R1H	1:1000
E-cadherin,	Cell Signaling Technology	3195	24E10	1:1000
Vimentin	Cell Signaling Technology	5741	D21H3	1:1000
Snail	Cell Signaling Technology	3879	C15D3	1:1000
Bax	Cell Signaling Technology	5023	D2E11	1:1000
Bcl-2	Cell Signaling Technology	3498	D17C4	1:1000
Cleaved Caspase-3	Cell Signaling Technology	9661	Asp175	1:1000
β -Actin	Cell Signaling Technology	8457	D6A8	1:1000
Anti-rabbit IgG	Cell Signaling Technology	7074		1:2000
Anti-mouse IgG	Cell Signaling Technology	7076		1:2000

(Cat. No: 5723S; Cell Signaling Technology, USA) in accordance with the manufacturer's instructions.

Immunofluorescence

Cells were grown on glass cover slips in a 24-well plate. After treatment, the cells were fixed with a solution containing 4% paraformaldehyde for 15 minutes. Subsequently, they were rinsed three times with PBS and permeabilized using 0.2% Triton X-100 at room temperature for 15 minutes. To prevent nonspecific binding, the cells were then blocked with a solution containing 5% bovine serum albumin for 1 hour. Following this, they were incubated overnight at a temperature of 4°C with the primary antibody specific to γ -H2AX (1:500; Cat. No: ab303656; Clone number: N1-431; Abcam), α B-crystallin (1:50; Cat. No: ab230722; Clone number: 6D11; Abcam), and Caspase-3 (1:25; Cat. No: 32351; Clone number: E87; Abcam). After washing the samples three times with PBST, the cover slips were incubated with fluorescent secondary antibodies: Anti-Rabbit IgG-Alexa Fluor 488 (1:500; Cat. No: ab150077; Abcam) or Anti-Mouse IgG-Alexa Fluor 647 (1:500; Cat. No: ab150115; Abcam) for 1 hour at room temperature in the dark. The samples underwent another round of washing thrice with PBST before being mounted using an anti-quenching reagent along with 4', 6-diamidino-2-phenylindole (Cat. No: P0131; Beyotime, China). Finally, foci representing γ -H2AX protein expression levels were quantified by examining at least six random fields under a Confocal Laser Scanning Microscope (FV1200; Olympus, Japan).

Co-Immunoprecipitation

Cells were lysed with a specialized buffer included in the Antibody Cross-linking IP/Co-IP Kit (Cat. No: P2180S; Beyotime, China) for subsequent immunoprecipitation analysis. And 50 μ L of total cell extracts were incubated overnight at 4°C with anti- α B-crystallin antibodies (1:20; Cat. No: ab230722; Clone number: 6D11; Abcam),

anti-Caspase-3 antibody (1:20; Cat. No: 32351; Clone number: E87; Abcam), or anti-IgG antibody (1:20; Clone number: EPR25A; Abcam). Subsequently, the antibody–protein complex was mixed with protein A/G plus-agarose beads and allowed to interact at room temperature for 2 h. The protein-coated beads were then separated and heated in a loading buffer. Finally, the precipitated protein complexes underwent western blot analysis.

Statistics

Graphs were generated and statistical analyses were conducted using GraphPad Prism 8.3.0 software. The data were assessed for normality using the Shapiro-Wilk test. Normally distributed data were compared between two groups using unpaired two-tailed t-tests. For non-normal data, the Wilcoxon rank test was used. Statistical significance was defined as $p \leq 0.05$. Each experiment was independently repeated at least three times, and all data are expressed as means $\pm$ standard deviation (SD).

Results

α B-crystallin facilitates cell migration in basal-like breast cancer cells

Most studies have shown that basal-like breast cancer exhibits highly aggressive biological behavior, is prone to early hematologic metastasis, and has low disease-free survival and overall survival. Therefore, α B-crystallin levels were determined in different breast cancer cell lines, including basal-like breast cancer cell lines (HCC1599, MDA-MB-468, MDA-MB-231) and luminal breast cancer cell lines (MDA-MB-453, BT-474, MCF-7). The results showed that α B-crystallin was highly expressed in basal-like cell lines compared to luminal cell lines (Figure 1(a)). The results demonstrated that the expression of α B-crystallin was significantly elevated in basal-like cell lines compared to luminal cell lines, with MDA-MB-231 cells exhibiting the highest expression and MCF-7 cells displaying the lowest levels. To further elucidate the role of α B-crystallin in breast cancer cells, we employed shRNA lentivirus particles to knock down α B-crystallin expression in MDA-MB-231 cells, while overexpressing it in MCF-7 cells through lentivirus-mediated delivery of α B-crystallin (Figure 1(b) and (c)). Transwell assays revealed that suppression of α B-crystallin in MDA-MB-231 cells significantly attenuated their invasive and migratory capacities compared to the control shRNA-treated cells (shNC). Conversely, overexpression of α B-crystallin in MCF-7 cells enhanced their migratory and invasive abilities when compared to the negative control (NC) (Figure 1(d)–(g)). Western blotting analysis confirmed the correlation between α B-crystallin and genes associated with epithelial-mesenchymal transition (EMT). The results demonstrated that, compared with the NC group, overexpression of α B-crystallin significantly upregulated the protein expression levels of mesenchymal markers N-cadherin and Vimentin, as well as the transcription factor Snail in MCF-7 cells (Figure 1(h) and (j)). Conversely, interfering with the expression of α B-crystallin in MDA-MB-231 cells markedly suppressed the expression of EMT-associated proteins

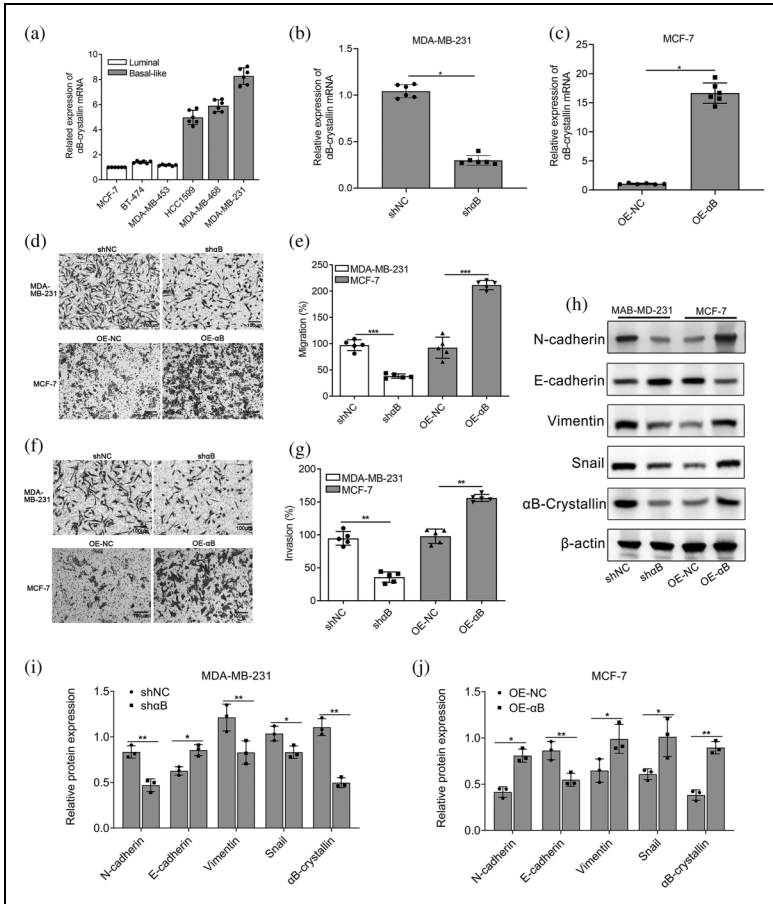


Figure 1. α B-crystallin facilitates cell migration in basal-like breast cancer cells. (a) Relative α B-crystallin expression levels in basal-like breast cancer cell lines HCC1599, MDA-MB-468, MDA-MB-231, and in luminal breast cancer cell lines MDA-MB-453, BT-474, MCF-7. (b) and (c) The expression levels of α B-crystallin in MDA-MB-231 and MCF-7 cells transfected with α B-crystallin knockdown or overexpression lentiviral vectors were quantified using qRT-PCR, with GAPDH serving as the internal loading control. Data are represented as means $\pm$ SD ($n = 6$; Wilcoxon rank test; $*p < 0.05$). (d)–(g): Migration (d) and (e) and invasion (f) and (g) activities of MDA-MB-231, MCF-7, and control cells were analyzed using a Transwell assay. Representative images (100 $\times$, Scale bar: 100 μ m) and summarized quantitative results are presented separately. Data are represented as means $\pm$ SD ($n = 5$; unpaired two-tailed Student's t -test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). (h)–(j) Western blot analysis of EMT markers (E-cadherin, N-cadherin, Vimentin, and Snail). β -Actin was utilized as the internal reference for normalization. The histogram presents the results of the quantitative analysis of the protein bands. Data are represented as means $\pm$ SD ($n = 3$; unpaired two-tailed Student's t -test; $*p < 0.05$, $**p < 0.01$). EMT: epithelial-mesenchymal transition; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; OE- α B: cells transfected with α B-crystallin overexpression vectors; qRT-PCR: quantitative real-time polymerase chain reaction; sh α B: cells transfected with α B-crystallin-specific shRNA; shNC and OE-NC: cells transfected with corresponding empty vectors.

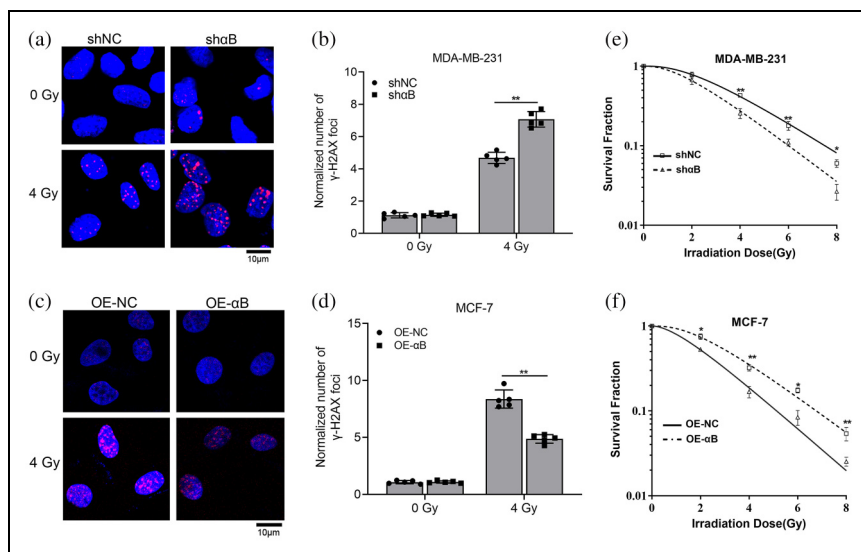


Figure 2. α B-crystallin enhances radioresistance in basal-like breast cancer cells. (a) and (c) Representative images of γ -H2AX foci formation were acquired in MDA-MB-231 + sh α B, MCF-7 + OE- α B, and control cells at 2-hour post 4 Gy of X-ray irradiation. Red fluorescence indicates γ -H2AX foci. These images were captured using a 60 $\times$ objective lens and subsequently magnified locally to facilitate detailed observation. Scale bar: 10 μ m. (b) and (d) The number of foci in each group was normalized to those in shNC or OE-NC cells from the 0 Gy group for quantification of γ -H2AX foci formation. (e) and (f) Colony formation and survival curve analysis were conducted on MDA-MB-231 + sh α B, MCF-7 + OE- α B, and control cells following exposure to varying doses of X-ray irradiation (0, 2, 4, 6, and 8 Gy). Data are represented as means $\pm$ SD ($n=5$; unpaired two-tailed Student's t -test; * $p<0.05$, ** $p<0.01$). OE- α B: cells transfected with α B-crystallin overexpression vectors; sh α B: cells transfected with α B-crystallin-specific shRNA; shNC and OE-NC: cells transfected with corresponding empty vectors.

compared to the shNC group (Figure 1(h) and (i)). These results strongly suggest that sufficient expression of α B-crystallin facilitates the metastasis of basal-like breast cancer cells.

α B-crystallin enhances radioresistance in basal-like breast cancer cells

The results demonstrated an increased number of γ -H2AX foci in α B-crystallin-silenced MDA-MB-231 cells compared to the control group following exposure to 4 Gy of X-rays (Figure 2(a) and (b)). Conversely, overexpression of α B-crystallin led to a reduction in γ -H2AX foci in irradiated MCF-7 cells (Figure 2(c) and (d)). The radiosensitization impact of α B-crystallin silencing in breast cancer cells was confirmed through a clonogenic assay. Results demonstrated that the colony survival fraction of MDA-MB-231 cells transfected with α B-crystallin-shRNA significantly decreased after X-ray irradiation compared to shNC cells (Figure 2(e)). Conversely, overexpression of α B-crystallin significantly enhanced the survival fraction of

MCF-7 cells irradiated by X-ray (Figure 2(f)). These findings suggest that downregulation of α B-crystallin enhances the radiosensitivity of basal-like breast cancer cells to X-ray irradiation.

Downregulation of α B-crystallin enhances radiation-induced apoptosis in basal-like breast cancer cells

Flow cytometry assay revealed that inhibition of α B-crystallin resulted in enhanced radiation-induced apoptosis in α B-crystallin-silenced MDA-MB-231 cells compared to control cells (Figure 3(a) and (b)). Conversely, overexpression of α B-crystallin significantly attenuated radiation-induced apoptosis in MCF-7 cells (Figure 3(c) and (d)). Similarly, in the radiation-treated MDA-MB-231 cells, compared with the control group, the knock-down of α B-crystallin resulted in increased levels of Bax and cleaved Caspase-3, as well as decreased expression of Bcl-2 (Figure 3(e) and (g)). Alternatively, overexpression of α B-crystallin in irradiated MCF-7 cells led to decreased levels of Bax and Cleaved Caspase-3, along with an increase in Bcl-2 expression (Figure 3(f) and (h)).

α B-crystallin interacts with Caspase-3 to attenuate its enzymatic activity

Recent studies have revealed an intriguing interaction between α B-crystallin and Caspase-3, a key enzyme involved in programmed cell death or apoptosis. It has been demonstrated that the binding of α B-crystallin to Caspase-3 inhibits the enzymatic activity of the latter, thereby preventing excessive apoptosis. This interaction highlights the importance of α B-crystallin as a protective factor against apoptotic processes. To determine whether α B-crystallin binds to Caspase-3 in breast cancer cells, we immunoprecipitated α B-crystallin from MDA-MB-231 and MCF-7 cells. As shown in Figure 4(a) and (b), α B-crystallin was found to co-immunoprecipitate with Caspase-3. To evaluate the effect of X-ray irradiation on the interaction between α B-crystallin and Caspase-3, an immunofluorescence assay was conducted. The results demonstrated a significant reduction in the interaction between α B-crystallin and Caspase-3 in MDA-MB-231 cells following α B-crystallin knockdown, whereas an enhanced interaction was observed in MCF-7 cells overexpressing α B-crystallin (Figure 4(c) and (d)). Similarly, the activities of Caspase-3 were evaluated, demonstrating that knockdown of α B-crystallin enhanced Caspase-3 activity in MDA-MB-231 cells, whereas overexpression of α B-crystallin decreased Caspase-3 activity in MCF-7 cells following 4 Gy X-ray irradiation (Figure 4(e) and (f)). These findings indicate that α B-crystallin binds to Caspase-3, thereby inhibiting its enzymatic activity and consequently modulating Caspase-3-mediated apoptosis.

Discussion

Breast cancer is one of the most prevalent types of cancer affecting women worldwide. Among its subtypes, basal-like breast cancer is known for its aggressive nature and poor

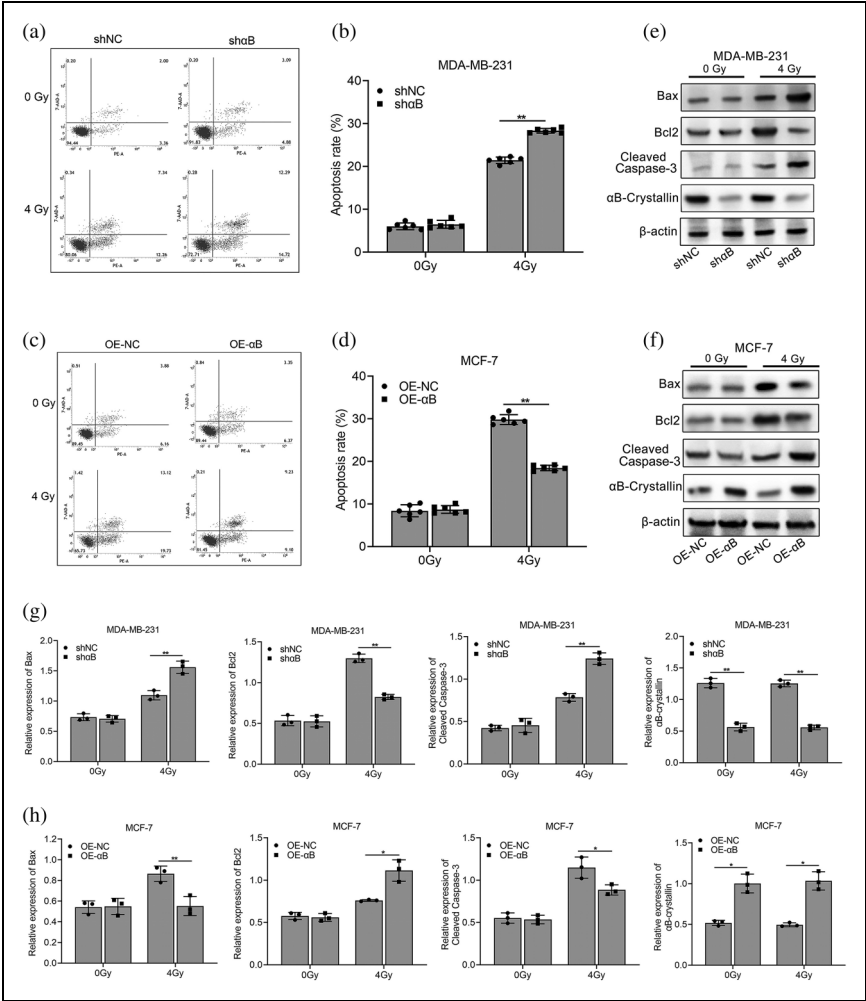


Figure 3. Downregulation of α B-crystallin enhances radiation-induced apoptosis in basal-like breast cancer cells. (a)–(d) The apoptosis rate was estimated using flow cytometry in MDA-MB-231 + sh α B, MCF-7 + OE- α B, and control cells at 48 h after exposure to 4 Gy of X-rays. Data are represented as means $\pm$ SD (n = 6; unpaired two-tailed Student's t-test; *p < 0.01). (e)–(h) Western blot analysis was performed to evaluate the expression levels of proteins associated with apoptosis at 48 hours after 4 Gy of X-ray irradiation. The histogram presents the results of the quantitative analysis of the protein bands. Data are represented as means $\pm$ SD (n = 3; unpaired two-tailed Student's t-test; *p < 0.05, **p < 0.01). OE- α B: cells transfected with α B-crystallin overexpression vectors; sh α B: cells transfected with α B-crystallin-specific shRNA; shNC and OE-NC: cells transfected with corresponding empty vectors.

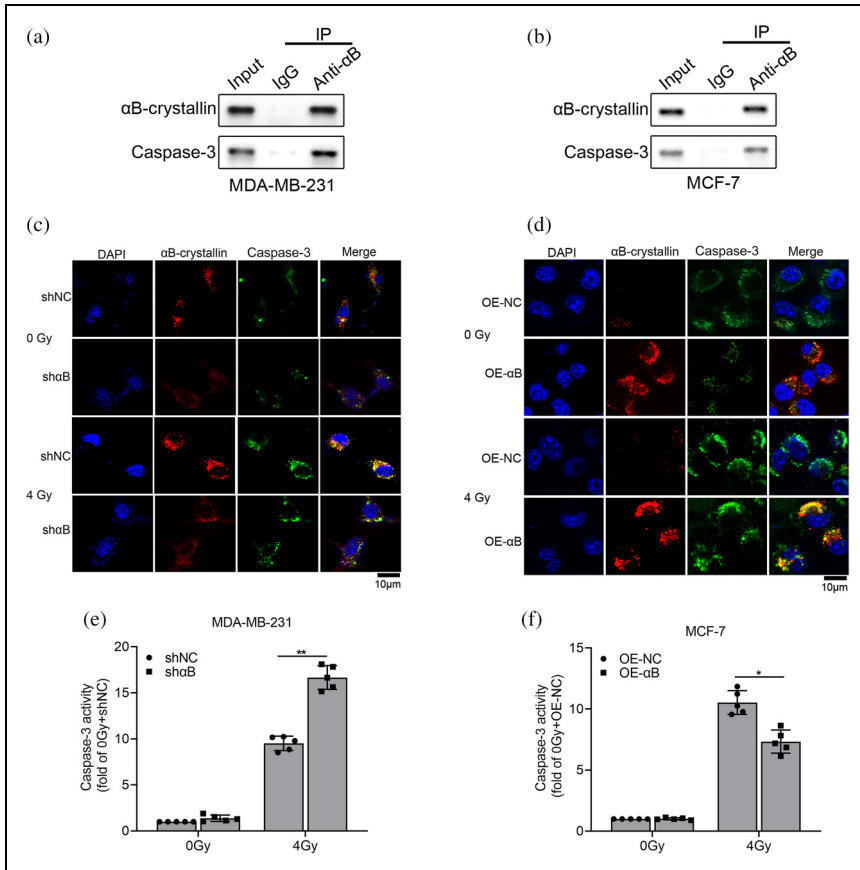


Figure 4. α B-crystallin interacts with Caspase-3 to attenuate its enzymatic activity. (a) and (b) Immunoprecipitation followed by western blot was used to detect the interaction of α B-crystallin with Caspase-3 in MDA-MB-231 and MCF-7 cells. (c) and (d) The co-localization of α B-crystallin (red) and Caspase-3 (green) in cells was detected using an immunofluorescence assay. Nuclei were stained with DAPI (blue). These images were acquired using a 60 $\times$ objective lens and subsequently magnified locally to facilitate detailed observation. Scale bar: 10 μ m. (e) and (f) The activities of Caspase-3 were assessed using Caspase-3 activity kits in MDA-MB-231 + sh α B, MCF-7 + OE- α B, and control cells at 48 hours post-exposure to 4 Gy of X-ray irradiation. Data are represented as means $\pm$ SD ($n = 5$; unpaired two-tailed Student's t -test; * $p < 0.05$, ** $p < 0.01$). DAPI: 4', 6-diamidino-2-phenylindole; OE- α B: cells transfected with α B-crystallin overexpression vectors; sh α B: cells transfected with α B-crystallin-specific shRNA; shNC and OE-NC: cells transfected with corresponding empty vectors.

prognosis. Understanding the molecular mechanisms underlying tumor progression and metastasis in this subtype is essential for developing effective therapeutic strategies. Recent studies have highlighted the involvement of α B-crystallin, a member of the small heat shock protein family, in facilitating cell migration and invasion in cancer cells.¹⁵ We

investigated the expression levels of α B-crystallin across common breast cancer cell lines and discovered that it was most prominently expressed in the basal-like breast cancer cell line MDA-MB-231, while exhibiting minimal expression in the luminal breast cancer cell line MCF-7. Compared with MCF-7 cells, MDA-MB-231 cells exhibited significantly stronger migration and invasion capabilities. However, the suppression of α B-crystallin markedly attenuated the migratory and invasive abilities of MDA-MB-231 cells, suggesting that excessive expression of α B-crystallin plays a critical role in promoting the migration and invasion of breast cancer cells. Mechanistically, α B-crystallin may play a role in epithelial-mesenchymal transition (EMT), a process that is closely associated with tumor metastasis. α B-crystallin induces EMT-associated transcription factors such as Snail and Slug through activation of signaling pathways like transforming growth factor beta/Smad or phosphoinositide 3-kinase/Akt/mechanistic target of rapamycin cascades.^{16–18} These transcription factors repress E-cadherin expression while upregulating mesenchymal markers like N-cadherin and vimentin, thereby enhancing invasive properties of basal-like breast cancer cells. The present study also revealed that knockdown of α B-crystallin significantly modulated the expression of proteins associated with EMT and effectively inhibited the migratory capacity of basal-like breast cells. Therefore, it is clinically viable to inhibit the expression of α B-crystallin to reduce metastasis and recurrence, thereby enhancing the therapeutic efficacy for breast cancer.

Radiotherapy serves as a primary modality for the conservative treatment of many cancers; however, the intrinsic radioresistance of cancer cells significantly diminishes its therapeutic efficacy. Basal-like breast cancer is characterized by its highly aggressive nature and resistance to conventional therapies, including radiotherapy. Radiotherapy functions by inducing DNA damage in tumor cells, thereby instigating their demise. However, α B-crystallin facilitates efficient repair of damaged DNA by interacting with other proteins involved in DNA repair pathways. This ultimately enables the survival and sustained proliferation of tumor cells despite exposure to radiation.^{19,20} We observed a significant reduction in radioresistance when α B-crystallin expression was downregulated in basal-like breast cancer cells. Conversely, the overexpression of α B-crystallin in highly radiosensitive luminal breast cancer cells led to a marked enhancement in radioresistance. However, radioresistant cells frequently evade apoptosis via multiple mechanisms. They might upregulate anti-apoptotic proteins such as Bcl-2 or downregulate pro-apoptotic factors like Bax, thereby preventing the activation of caspases, which are key enzymes in the execution of apoptosis. α B-crystallin exhibits an anti-apoptotic effect on basal-like breast cancer cells following irradiation, thereby suppressing programmed cell death initiated by severe cellular stress or damage caused by radiotherapy.^{21,22} This study demonstrated that the downregulation of α B-crystallin augmented DNA damage and apoptosis in basal-like breast cancer cells induced by radiation. Further research aimed at developing targeted strategies or inhibitors against α B-crystallin could potentially enhance the efficacy of radiotherapy for basal-like breast cancer.

By inhibiting apoptosis signaling pathways, α B-crystallin prevents the programmed cell death of irradiated tumor cells and promotes their survival instead. Elucidating the mechanisms by which α B-crystallin enhances radioresistance in basal-like breast cancer

provides critical insights into potential therapeutic targets, thereby facilitating the optimization of treatment strategies for patients with this aggressive subtype. α B-crystallin has been identified as a pivotal regulator of Caspase-3 enzymatic activity, which is crucial for programmed cell death or apoptosis. By interacting with Caspase-3, α B-crystallin effectively functions as a potent inhibitor, thereby attenuating its enzymatic activity.²³ The interaction between α B-crystallin and Caspase-3 functions as a protective mechanism against excessive cellular apoptosis, thereby contributing to the maintenance of cellular homeostasis and prevention of unnecessary cell loss.²⁴ Additionally, the present research demonstrated that the interaction between α B-crystallin and Caspase-3 not only attenuates its enzymatic activity but also modulates its substrate specificity, thereby implying a potential influence of α B-crystallin on downstream signaling pathways regulated by activated Caspase-3. However, the precise molecular mechanism underlying the downstream effects of the interaction between α B-crystallin and Caspase-3 has not been fully elucidated. Ongoing research is being conducted to address this knowledge gap. It is hypothesized that specific regions or domains within both proteins play a crucial role in their binding affinity and subsequent modulation of Caspase-3 activity.²⁵ Comprehending these structural intricacies has the potential to pave the way for therapeutic strategies targeting this interaction in diverse pathologies characterized by aberrant apoptosis. However, α B-crystallin is expressed not only in cancer cells but also in other normal cells, such as lens cells. Therefore, specifically inhibiting α B-crystallin in tumor cells while preserving its function in normal cells will be a significant challenge and an important research direction for our future studies.

Conclusion

In summary, our findings indicate that α B-crystallin enhances the radioresistance of basal-like breast cancer cells through its interaction with Caspase-3, thereby inhibiting Caspase-3-mediated apoptosis. This interaction between α B-crystallin and Caspase-3 offers valuable insights into the intricate regulatory network governing radiation-induced apoptosis. Further investigation into the complex interplay between these two proteins promises significant advancements in our understanding of apoptotic pathways and may lead to the identification of novel therapeutic strategies for basal-like breast cancer.

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data availability statement

The datasets utilized and/or examined in this study can be obtained from the corresponding author upon reasonable request.

Supplemental material

Supplemental material for this article is available online.

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