B7-H3 upregulates BRCC3 expression, antagonizing DNA damage caused by 5-Fu

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Abstract. 5-Fluorouracil (5-Fu) is still recognized as the mainstay in colorectal cancer chemotherapy, but the response rate of 5-Fu in colorectal cancer is less than 50%. Our previous mRNA microarray data revealed that BRCC3, a component of the BRCA1-BRCA2-BRCC3 DNA repair complex, had a direct relationship with B7-H3, an immunoglobulin that is upregulated in tumor tissue and associated with metastasis and poor prognosis. Real-time PCR and western blot analysis confirmed that the expression of both BRCC3 mRNA and protein, respectively, were elevated following B7-H3 overexpression in SW480 cells; likewise, BRCC3 expression decreased after B7-H3 was knocked down in HCT-8 cells. DNA comet assay results indicate an inverse correlation between the extent of 5-Fu-induced DNA damage and the expression level of B7-H3 in both SW480- and HCT-8-based cell lines. In SW480 cells that overexpress B7-H3, knockdown of BRCC3 similarly permitted greater 5-Fu-induced DNA damage. Altogether, results suggest that BRCC3 may play a role in B7-H3-induced 5-Fu resistance, such that B7-H3 upregulates BRCC3 expression, enhancing DNA repair in colorectal cancer cells.

Introduction

Colorectal cancer (CRC) is one of the three leading causes of cancer-related deaths worldwide. Despite rapidly developing novel targeted therapies, 5-fluorouracil (5-Fu) is still recognized as the mainstay in colorectal cancer chemotherapy. However, the response rate to 5-Fu in colorectal cancer adjuvant chemotherapy is less than 50%, and recurrence of colon cancer occurs in almost 50% of patients treated by standard therapeutics, according to NCCN (National Comprehensive Cancer Network) (1). Resistance to 5-Fu has been attributed, at least in part, to enhance DNA repair.

The B7 family is an immunoglobulin superfamily, which consists of B7-1, B7-2, B7-H1, B7-H2, B7-DC, B7-H3, B7-H4, B7-H5 and B7-H6 (2). As key immunity modulating molecules, the B7 family is important for the treatment of cancer, transplantation and autoimmune diseases (3). B7-H3 was first identified in monocytes and dendritic cells (4), which regulate T cell functions, including proliferation, apoptosis and adhesion. B7-H3 is also overexpressed in tumor tissue, where it is associated with cancer metastasis and poor prognosis (5-9), suggesting a role for B7-H3 in tumor progression (8). Our previous results indicated that overexpressed B7-H3 significantly increased anti-apoptosis and the metastasis ability of CRC cells.

According to our mRNA microarray data, increasing the expression of B7-H3 induced a subsequent increase in BRCC3 expression. BRCC3 and BRCA1/BRCA2 are known to form a complex that repairs DNA damage (10). Downregulation of BRCC3 causes glioma to become more sensitive to chemotherapy (11). The roles of BRCA1 and BRCA2 in breast, ovarian, and other cancer have been reported in many clinical and experimental studies (12,13). Results indicate that the BRCA1/BRCA2/BRCC3 DNA damage repair complex is closely related to tumor recurrence and metastasis. In the present study, we focused on the relationship between B7-H3 and BRCC3 to investigate the mechanism of B7-H3-induced 5-Fu resistance due to enhanced DNA repair.

Materials and methods

Cells and reagents. Two human CRC cell lines, SW480 and HCT-8, exhibit different expression levels of B7-H3. We

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constructed SW480 cells that expressed high levels of B7-H3 (SW480-B7-H3) and HCT-8 cells that were stably transfected with B7-H3 shRNA (HCT-8-shB7-H3). Cells transfected with a mock vector were used as negative controls (SW480-NC and HCT-8-NC). Cells were maintained in Dulbecco's modified Eagle's medium or RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), at 37°C in a humidified atmosphere with 5% CO_2.

5-fluorouracil (5-Fu) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-human B7-H3, β-actin and BRCC3 antibodies were purchased from Abcam (Cambridge, MA, USA); the α-tubulin antibody was purchased from Biotime (Shanghai, China); the horseradish peroxidase-conjugated secondary anti-rabbit antibodies were purchased from Beyotime Institute of Biotechnology (Nantong, China); the secondary antibodies fluorophore-labeled donkey anti-rabbit IgG (A11374) were purchased from Invitrogen (Carlsbad, CA, USA).

CCK-8 assay. SW480-NC/SW480-B7-H3 or HCT-8-NC/HCT-8-shB7-H3 cells were seeded at 3,000 cells/well in 96-well plates in triplicate. The next day, the medium was replaced with medium containing 5-Fu at a 2-fold concentration gradient. After 48 h, 10 µl CCK-8 was added to each well, and the absorbance was recorded on a BioTek microplate reader at the wavelength of 450 nm. Cell viability was assessed in percent wherein vehicle-treated cells were taken as 100%. Half maximal inhibitory concentration (IC_{50}) was calculated using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Real-time quantitative polymerase chain reaction. Total RNA was isolated from $1.5x10^6$ cells using TRIzol, following the manufacturer's instructions, and then treated with RNase-free DNase to remove residual genomic DNA. The first strand cDNA was synthesized from 1 µg RNA using a SuperScript First-Strand Synthesis system (Invitrogen). First-strand cDNA was amplified in a 20 µl PCR reaction mixture: 10 µl 2X SYBR-Green PCR Master Mix, 0.4 µl 50X ROX, 0.4 µl of each specific primer set and ddH_2O added to 20 µl. The sequences of primers were as follows: β-actin 5'-AGCGAGC ATCCCCCAAAGTT-3' (sense) and 5'-GGGCACGAAGGC TCATCATT-3' (antisense); BRCC3 5'-AGAGTTCAGAGTA TGAGAGAATCG-3' (sense) and 5'-TCTTGGTTACTGAGT CCAGATGT-3' (antisense); The PCR cycling consisted of 40 cycles of amplification of the cDNA with annealing at 60°C.

Western blot assay. Western blot assays were performed on whole-cell extracts prepared by lysing $1x10^6$ cells containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and PMSF protease inhibitor. Equal protein loading of the lysates was achieved by standardization with the BCA protein assay kit from Biotime. Samples were separated on SDS-PAGE gels and transferred to nitrocellulose transfer membranes. After blocking with 5% skim milk in TBST, the membranes were incubated with the appropriate primary antibody (α-tubulin or BRCC3) overnight at 4°C, followed by incubation with HRP-conjugated secondary antibody for 2 h at room temperature. The protein bands were visualized with an enhanced chemiluminescence.

Immunofluorescence. Cells were fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 for 10 min at room temperature (RT). Cells were washed three times in PBS after each treatment. After blocking with 2% BSA in PBS for 30 min at room temperature, the cells were incubated with primary antibody in 2% BSA/PBS overnight at 4°C and then incubated with fluorescent secondary antibody for 1 h at room temperature. Cell nuclei were stained with DAPI for 10 min at RT. Images were acquired with the TSC SP8 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany).

Comet assay. The comet assay was performed under alkaline conditions according to Singth *et al* (14). Cells were seeded in 12-well tissue-culture plates and incubated for 24 h for cell attachment. Subsequently, cells were treated with increasing concentrations of 5-Fu (positive control) for 24 h. Cells were harvested, washed and resuspended in ice-cold PBS. Approximately 25 µl of the resuspended cells were mixed with 75 µl of low melting point agarose at 37°C; the suspension was spread over the well with the pipette tip. The slides were placed at 4°C in the dark until gelling occurred and then were immersed in pre-chilled lysis buffer at 4°C. After incubating for 2 h, the buffer was aspirated and replaced with pre-chilled alkaline solution for 30 min at 4°C. After lysis and unwinding, the slides were placed in a horizontal electrophoresis tank filled with freshly prepared alkaline electrophoresis buffer. The electrophoresis was run at 20 V and 200 mA for 30 min. After electrophoresis, the slides were transferred to pre-chilled distilled water for 2 min, then aspirated and repeated twice. The final rinse was aspirated and replaced with cold 70% ethanol for 5 min. Thereafter, the slides were allowed to air dry, and 100 µl/well of PI was added to each slide for 20 min in the dark at room temperature for DNA staining. DNA migration was observed using a Nikon 90i fluorescence microscope. For each concentration, 6-10 randomly selected cells were analyzed. All of the comet images were analyzed using CASP software (CASPlab, Wroclaw, Poland) (15), and the percentage of DNA in the comet tail (TDNA%), tail length (TL), tail moment (TM) and olive tail moment (OTM) were recorded to characterize the cell DNA damage. All the experiments were repeated once, and the variation between experiments was analyzed.

Colony formation assay. Colony formation assays were performed by replating cells at a density of 300 cells/well onto a 6-well culture plate in medium containing 10% FBS. After 3 weeks, the cells were fixed with ice-cold methanol for 10 min and then stained by using 0.05% crystal violet in ddH_2O for 15 min. Alternatively, for examination of clonogenic ability of SW480 and HCT-8 cells with 5-Fu treatment, a density of 600 cells/well were seeded onto a 6-well culture plate in medium containing 10% FBS and 2 µg/ml 5-Fu or vehicle was added to the cultures at day 3 after seeding. The cultures were continuously maintained for another 2 weeks and subjected to the colony formation assay. Colonies produced by each cell-group were counted and measured using ImageJ software.

Knockdown of BRCC3 by small interfering RNA (siRNA). siRNA against BRCC3 were chemically synthesized by
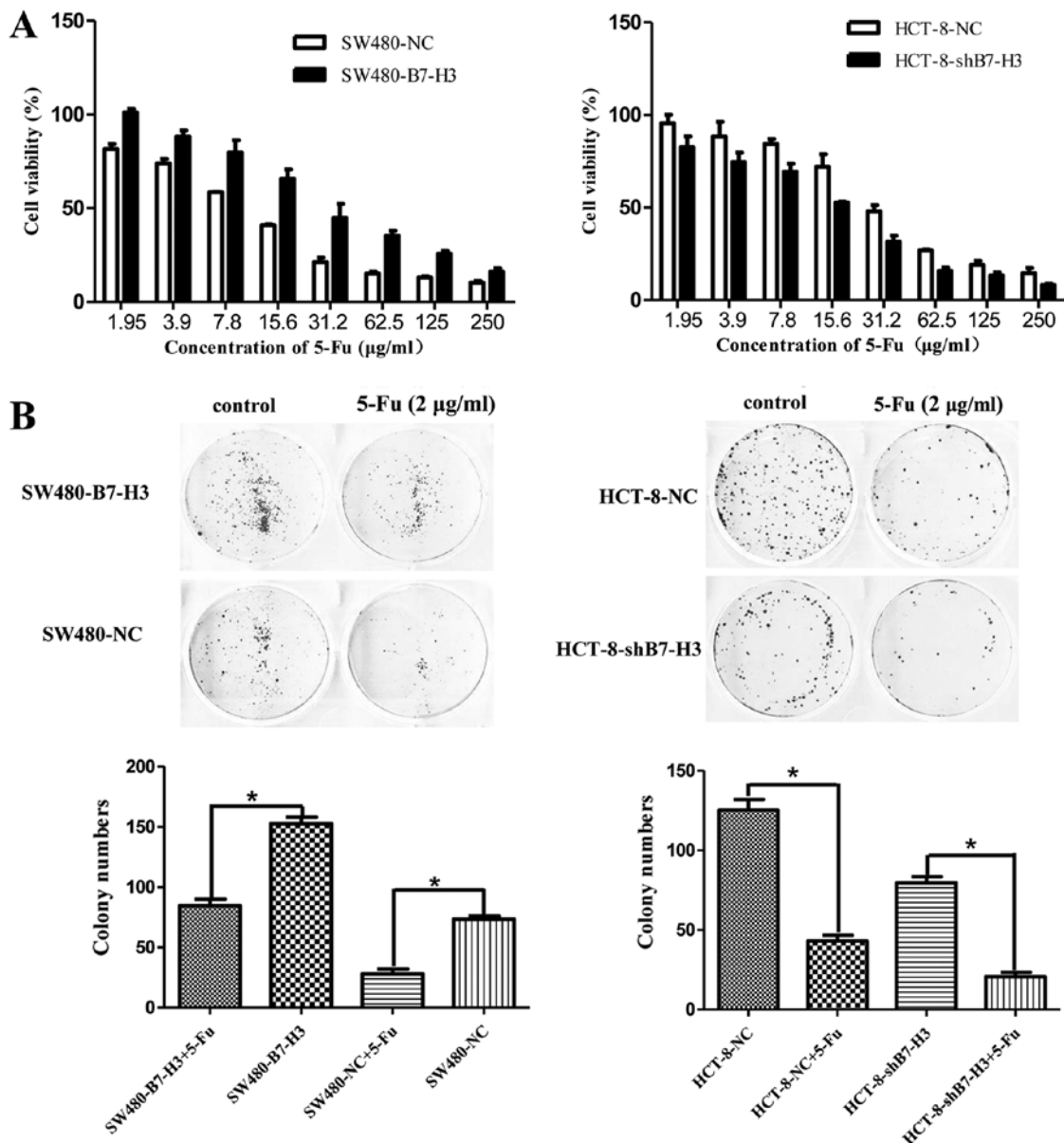
Figure 1. Overexpression of B7-H3 attenuated the sensitivity of CRC cells to 5-Fu. (A) Stably transfected SW480 and HCT-8 cells were incubated with different concentrations of 5-Fu for 48 h; the IC_{50} of the effect of 5-Fu on cell viability was derived using the CCK-8 assay; (B) colony formation assay. Data are presented as mean ± SEM of three independent experiments; [*]$P<0.05$. 5-Fu, 5-fluorouracil; NC, negative control.

RiboBio Co., Ltd. (Guangzhou, China). Transfection of siRNAs was performed using the Oligofectamine reagent (Invitrogen). The sequences of siRNA-BRCC3 are as follows: siRNA1-GCAGGAATTACAACAAGAA, siRNA2-GAAGGACCGAGTAGAAATT.

Statistical analyses. All experiments were re-performed at least three times with similar results. Quantitative data were presented as the average value of replicates ± SD (standard deviation) within the representative experiment.

Results

Overexpression of B7-H3 weakens the sensitivity of CRC cells to 5-Fu. To inhibit tumor growth, we incubated SW480-NC, SW480-B7-H3, HCT-8-NC, or HCT-8-shB7-H3 cells with a concentration gradient of 5-Fu for 48 h (Fig. 1A). CCK-8

assays showed that the inhibition rate of SW480-B7-H3 was less than SW480-NC at any concentration of 5-Fu ($P<0.05$). The IC_{50} of 5-Fu increased from 10.58 to 32.46 μg/ml after B7-H3 was upregulated. The HCT-8 pairs showed similar results ($P<0.05$): the IC_{50} of 5-Fu decreased from 31.28 to 14.87 μg/ml after B7-H3 was knocked down. We next studied the ability of cancer cells to form colonies on 6-well plates in presence or absence of 5-Fu. Consistent with the CCK-8 assay results, the colony formation of cells overexpressing B7-H3 (SW480-B7-H3 and HCT-8-NC) was increased compared with the cells with low expression of B7-H3 (SW480-NC and HCT-8-shB7-H3) (Fig. 1B). Therefore, these observations demonstrate that the overexpression of B7-H3 weakened the sensitivity of CRC cells to 5-Fu.

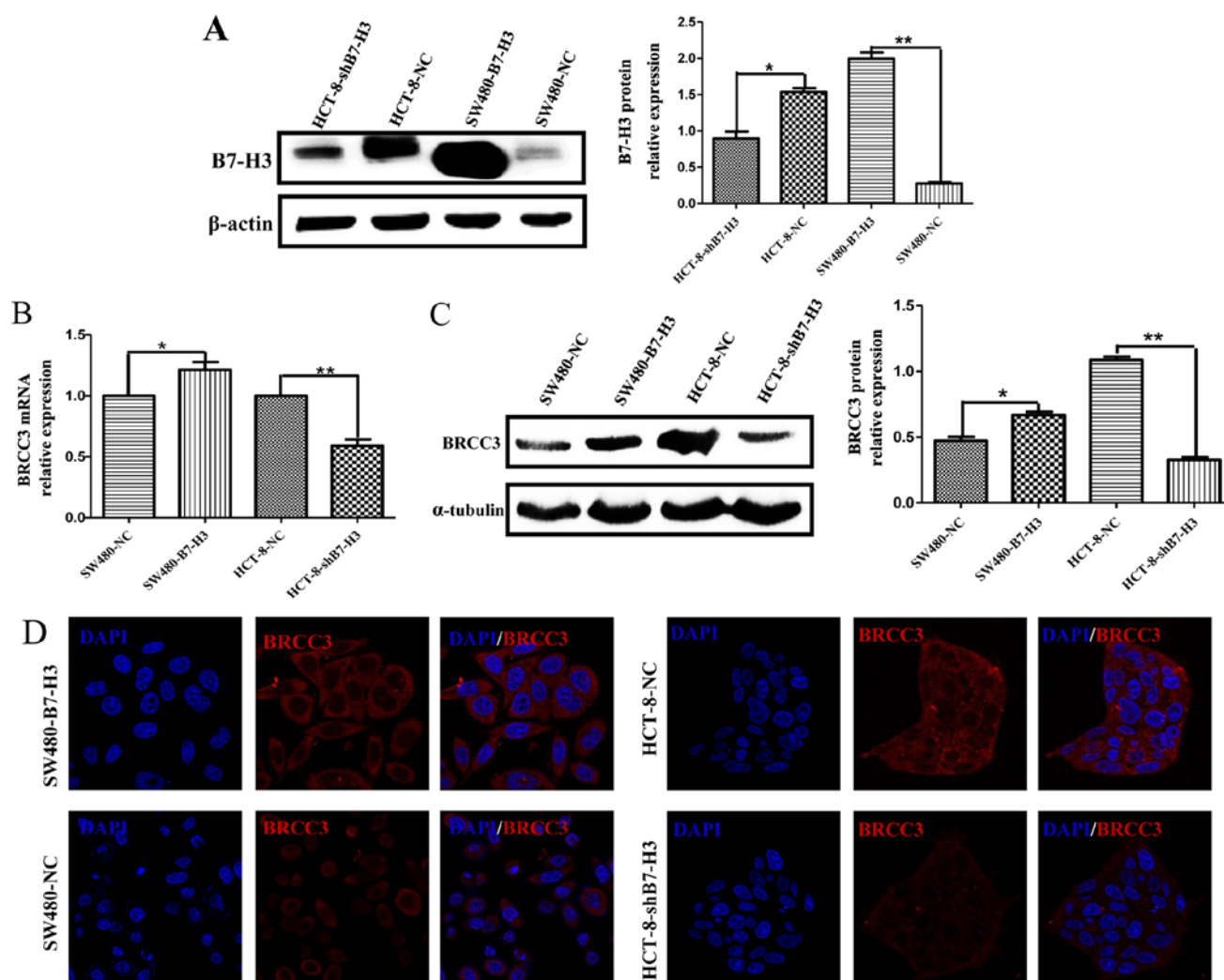*Overexpression of B7-H3 upregulated BRCC3 expression in CRC cells.* The mechanism by which B7-H3 induces resis-

Figure 2. Overexpression of B7-H3 upregulates BRCC3 expression in CRC cells. (A) Total protein was isolated from two pairs of cells, and then subjected to western blot analysis for the measurement of B7-H3 protein levels. (B) Real-time PCR, (C) western blot analysis and (D) immunofluorescent staining showed higher BRCC3 mRNA and protein expression in SW480-B7-H3 and HCT-8-NC cells than in low-B7-H3-expressing cells (SW480-NC and HCT-8-shB7-H3 cells). Data are presented as mean ± SEM of three independent experiments. *P<0.05, **P<0.01.
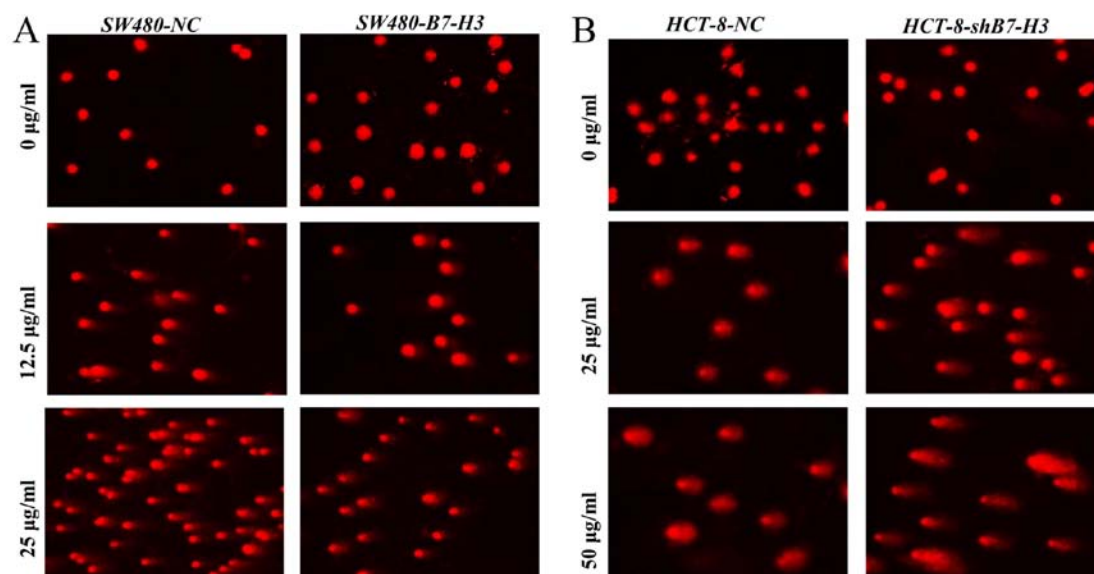

Figure 3. Overexpression of B7-H3 suppresses 5-Fu-induced DNA damage in CRC cells. SW480 cell pairs (A) were treated with 5-Fu (0, 12.5 and 25 µg/ml) and HCT-8 cell pairs (B) were treated with 5-Fu (0, 25 and 50 µg/ml) for 24 h, then cells were harvested, and DNA damage was determined by comet assay. Data are presented as mean ± SEM of three independent experiments.

Table I. SW480-NC and SW480-B7-H3 cells were treated with 5-Fu for 24 h, DNA damage was measured by DNA comet assay.

Group	0 μg/ml-5-Fu		12.5 μg/ml-5-Fu		25 μg/ml-5-Fu	
	SW480-NC	SW480-B7-H3	SW480-NC	SW480-B7-H3	SW480-NC	SW480-B7-H3
TDNA%	1.8±1.2	0.2±0.4[a]	21.3±3.8	12.5±4.7[b]	33.1±9.8	22.9±4.1[a]
TL	4.4±1.2	3.0±0.0[a]	24.1±5.6	21.1±3.0[a]	30.7±7.1	26.3±4.0
TM	0.1±0.1	0.0±0.0	5.3±1.9	2.8±1.4[b]	10.7±6.2	6.1±1.8
OTM	0.2±0.2	0.0±0.0	3.7±1.0	3.0±1.3	6.4±2.8	4.8±1.1

The TDNA%, TL, TM and OTM were measured in each cell line; data are presented as mean ± SD from at least 6-10 cells in each treatment group. TDNA%, tail DNA%; TL, tail length; TM, tail moment; OTM, olive tail moment; 5-Fu, 5-fluorouracil. [a]P<0.05 and [b]P<0.001, SW480-B7-H3 *vs*. SW480-NC.

Table II. HCT-8-NC and HCT-8-shB7-H3 cells were treated with 5-Fu for 24 h, DNA damage was measured by DNA comet assay.

Group	0 μg/ml-5-Fu		25 μg/ml-5-Fu		50 μg/ml-5-Fu	
	HCT-8-NC	HCT-8-shB7-H3	HCT-8-NC	HCT-8-shB7-H3	HCT-8-NC	HCT-8-shB7-H3
TDNA%	0.9±0.7	1.3±1.4	16.1±4.4	31.1±8.0[a]	27.4±10.3	60.6±8.9[a]
TL	3.6±1.1	3.6±1.1	15.6±2.3	36.3±6.2[a]	22.7±5.3	55.2±7.0[a]
TM	0.0±0.0	0.1±0.1	2.6±0.9	12.0±4.3[a]	6.7±3.6	34.0±9.0[a]
OTM	0.1±0.1	0.2±0.2	3.1±0.8	7.5±2.4[a]	6.4±2.2	18.0±4.4[a]

The TDNA%, TL, TM and OTM were measured in each cell line; data are presented as mean ± SD from at least 6-10 cells in each treatment group. TDNA%, tail DNA%; TL, tail length; TM, tail moment; OTM, olive tail moment; 5-Fu, 5-fluorouracil. [a]P<0.001, HCT-8-shB7-H3 *vs*. HCT-8-NC.

tance to 5-Fu in cancer cells is very complicated. We have previously reported that B7-H3 could enhance cancer cell resistance to apoptosis by increasing the expression of anti-apoptotic proteins Bcl-2 and Bcl-xl. Here, we examined the expression of B7-H3 in stably transfected CRC cells using western blot analysis (Fig. 2A) and analyzed BRCC3 expression in response to B7-H3 overexpression or knockdown using real-time PCR (Fig. 2B), western blot analysis (Fig. 2C) and immunofluorescence (Fig. 2D). Both mRNA and protein levels of BRCC3 in SW480-NC, SW480-B7-H3, HCT-8-NC, and HCT-8-shB7-H3 cells corresponded with B7-H3 expression, such that B7-H3-overexpressing cells (SW480-B7-H3 and HCT-8-NC) demonstrated higher BRCC3 expression compared to cells having low B7-H3 expression (SW480-NC and HCT-8-shB7-H3). Immunofluorescence showed that BRCC3 expression was predominantly cytosolic.

Overexpression of B7-H3 suppresses DNA damage of CRC cells by 5-Fu. Since BRCC3 has been reported to be involved in mediating DNA repair (16), we examined its role in repairing 5-Fu-induced DNA damage in CRC cells (Fig. 3). SW480-NC and SW480-B7-H3 cells were treated with 5-Fu (0, 12.5 and 25 μg/ml; Fig. 3A), and HCT-8-NC and HCT-8-shB7-H3 cells were treated with 5-Fu (0, 25 and 50 μg/ml; Fig. 3B) for 24 h; subsequent DNA damage was measured by DNA comet assay. As depicted, treatment with 12.5 or 25 μg/ml 5-Fu induced

Table III. SW480-B7-H3 cells, transfected with siRNA targeting BRCC3 or with scramble siRNA, were treated with 5-Fu for 24 h, DNA damage was measured by DNA comet assay.

Group	12.5 μg/ml-5-Fu	
	SW480-B7-H3/ scramble	SW480-B7-H3/ BRCC3-siRNA1
TDNA%	7.7±3.9	21.2±6.4[a]
TL	25.0±8.9	56.7±15.7[a]
TM	2.2±1.4	12.9±6.0[a]
OTM	3.7±1.8	12.0±3.4[a]

The TDNA%, TL, TM and OTM were measured. Data are presented as mean ± SD from at least 6-10 cells in each treatment group. TDNA%, tail DNA%; TL, tail length; TM, tail moment; OTM, olive tail moment; 5-Fu, 5-fluorouracil. [a]P<0.001, SW480-B7-H3/BRCC3-siRNA1 *vs*. SW480-B7-H3/scramble.

longer DNA tails and higher tail-DNA content in SW480-NC versus SW480-B7-H3 cells, indicating greater DNA damage in cells expressing less B7-H3. Similarly, treatment with 25 or 50 μg/ml 5-Fu induced longer DNA tails and higher tail-
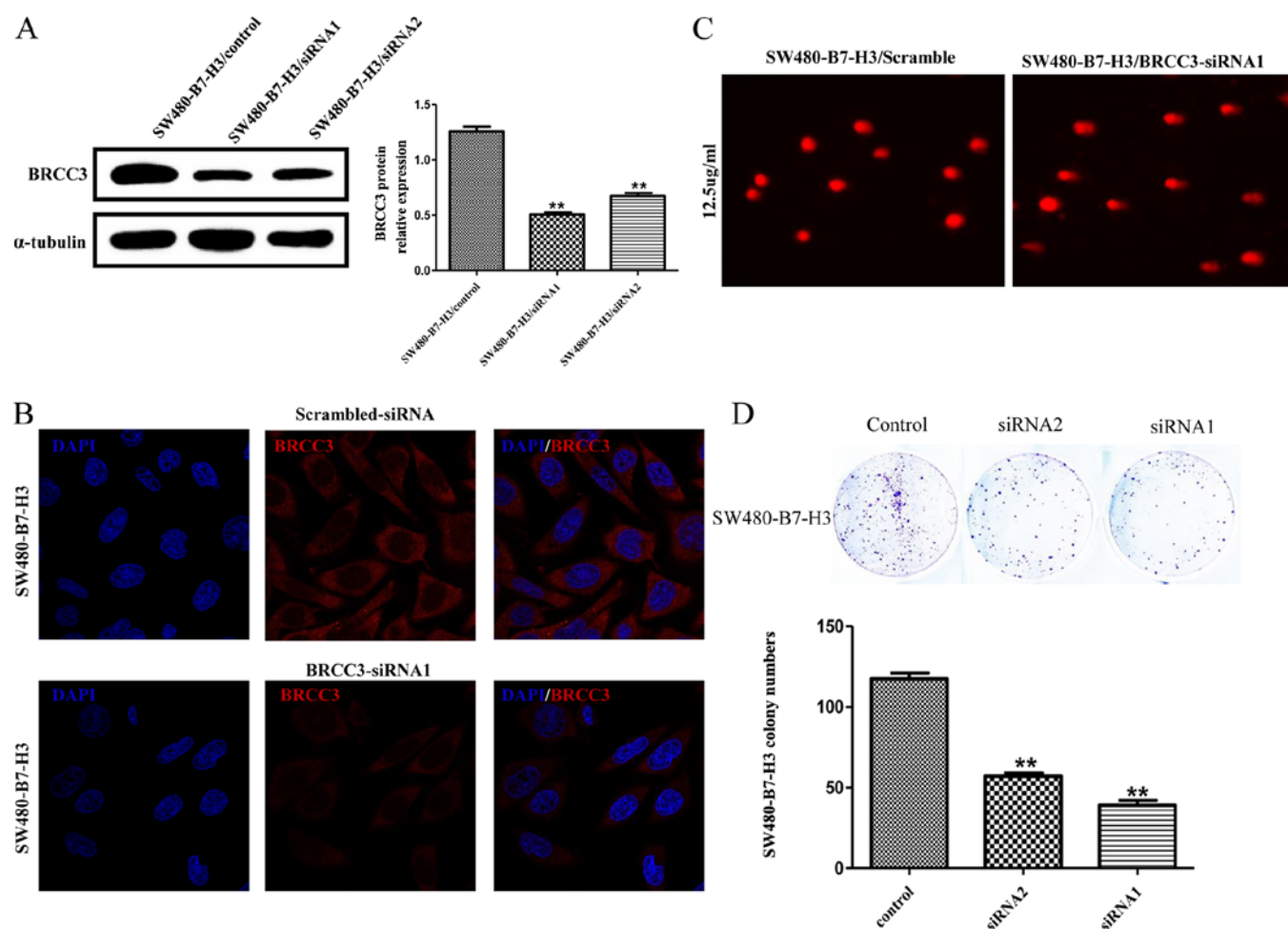
Figure 4. Downregulated BRCC3 expression blocks B7-H3. (A) Total protein was isolated from SW480-B7-H3 cells transfected with BRCC3-siRNA1 and BRCC3-siRNA2, and then subjected to western blot analysis for the measurement of BRCC3 protein levels. α-tubulin served as a domestic loading control. (B) Representative immunofluorescence image of SW480-B7-H3 cells stained with BRCC3 (red) and DAPI (blue) after cells were transfected with BRCC3 or scramble siRNA. (C) SW480-B7-H3 cells, transfected with BRCC3 or scramble siRNA, were treated with 5-Fu (12.5 µg/ml) for 24 h; DNA damage was determined using the comet assay. (D) Colony formation assay. Images were taken after three weeks. Experiments were repeated twice, and mean ± SEM are shown. **P<0.01.

DNA content in HCT-8-shB7-H3 *vs.* HCT-8-NC cells. Percent TDNA, TL, TM and OTM were quantitatively analyzed by CASP software; results are listed in Tables I (corresponding to Fig. 3A) and II (corresponding to Fig. 3B).

Downregulated expression of BRCC3 blocks B7-H3. Fig. 4A depicts western blot analysis demonstrating the effect of knocking down BRCC3 protein expression with either siRNA1 or siRNA2 (two different siRNA sequences targeting BRCC3); representative immunofluorescence images depicting BRCC3 knockdown are shown in Fig. 4B.

The DNA comet assay shown in Fig. 4C indicates that 5-Fu (25 µg/ml) elicits greater DNA damage in SW480-B7-H3 cells transfected with BRCC3 siRNA1 *vs.* scramble control siRNA (Fig. 4C). The TDNA%, TL, TM and OTM results are recorded in Table III. Further results indicated that knocking down BRCC3 expression in SW480-B7-H3 cells effectively suppressed colony formation (Fig. 4D). Collectively, our data showed that downregulated BRCC3 can induce DNA damage and reduce colony formation, thereby antagonizing two characteristics of B7-H3. These results suggest that BRCC3 may

play a key role in 5-Fu resistance and DNA repair activation caused by B7-H3.

Discussion

The present study first demonstrated that B7-H3 could upregulate BRCC3 expression to increase the ability to repair DNA and to antagonize DNA damage caused by 5-Fu. We and other researchers have discovered that co-stimulatory molecule B7-H3 could cause cancer cells resistance to 5-Fu or other agents, and reported few probable mechanisms to explain this phenomenon; for example, B7-H3 has been shown to induce Bcl-2 and Bcl-xl overexpression via the Jak2-STAT3 signaling pathway to inhibit cancer cell apoptosis (17). Here, we propose that B7-H3 may enhance DNA repair to attenuate DNA damage induced by chemotherapy medications.

5-Fu and its derivatives are recognized as the cornerstone pharmaceuticals in gastrointestinal cancer chemotherapy. The classic chemotherapy regimens FOLFOX and FOLFIRI were both developed from 5-Fu. 5-Fu, a uracil analog, blocks synthesis of the pyrimidine thymidine, interrupting DNA
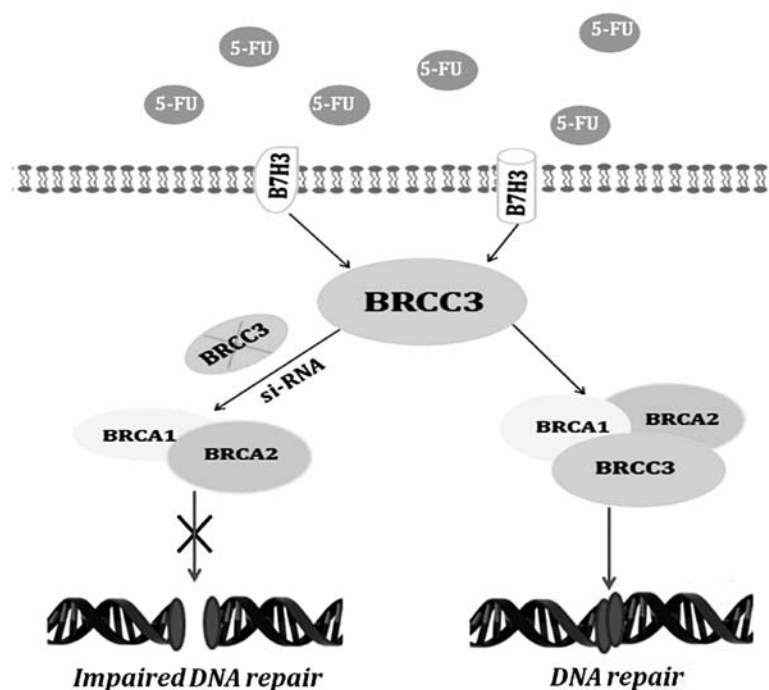
Figure 5. B7-H3 antagonizes DNA damage caused by 5-Fu through upregulating BRCC3 in colorectal cancer. According to this model, overexpression of B7-H3 upregulates BRCC3, and together with other DNA repair-associated molecules, BRCC3 promotes effective DNA damage repair, contributing to 5-Fu resistance in CRC cells.

replication. 5-Fu also antagonizes homologous recombination repair, leading to persistent DNA damage (18). Therefore, we analyzed 5-Fu resistance in colorectal cancer cells after upregulating or downregulating B7-H3.

Our experiments showed that overexpressing B7-H3 in SW480 cells lead to increased resistance to 5-Fu; similarly, HCT-8 cells became more sensitive to 5-Fu after B7-H3 had been knocked down. The relationship between B7-H3 expression and an increased 5-Fu IC_{50} may be due to the B7-H3 induction of the Bcl-2 and Bcl-xl anti-apoptotic proteins, as previously presented, in addition to its induction of the DNA repair complex protein BRCC3, as presented here.

According to our previous mRNA microarray results, we discovered that the expression of BRCC3 fluctuated following changes in B7-H3 expression. Confirmation of these results using both real-time PCR to measure mRNA levels and western blot analysis to determine protein levels underscored a regulatory relationship between B7-H3 and BRCC3. However, little has been reported regarding the regulation of BRCC3 expression and B7-H3 signal transduction (17). Thus, the detail mechanism between B7-H3 and BRCC3 need more projects to be elucidated.

Comet analysis is a classic method to measure DNA damage: The length and densities of the comet tails have been used to quantify the extent of DNA damage. Comet analysis of SW480-B7-H3 vs. SW480-NC cells after 24-h treatment with 12.5 or 25 µg/ml 5-Fu indicated less DNA damage in cells expressing higher levels of B7-H3, consistent with the hypothesis that B7-H3 induces BRCC3 expression, which enhances DNA repair. In this way, B7-H3 and BRCC3 expression can be thought to antagonize the effects of 5-Fu. In order to test this hypothesis, we transiently transfected BRCC3 siRNA into SW480-B7-H3 cells. Results showed that knocking down BRCC3 expression attenuated DNA damage repair. Altogether, comet analyses indicated that downregulated BRCC3 (SW480-B7-H3 cells) or downregulated B7-H3 expression (SW480-NC vs. SW480-B7-H3 cells or HCT-8-shB7-H3 vs. HCT-8-NC cells) resulted in increased DNA damage, presumably due to attenuated DNA repair.

Although B7-H3 was first identified as an immunity checkpoint molecule, recently studies, including ours, have focused on it due to its abnormal expression in malignant tumors and its association with poor prognosis. A false subcellular location of B7-H3 is also observed in colorectal cancer patients. Previous in vivo experiments implied that B7-H3 could activate Jak2-STAT3, PI3K-Akt pathway to subsequently induce the expression of Bcl-2, Bcl-xl and MMP9 (17,19). In the present study, we have shown that B7-H3 can upregulate the DNA repair process in cancer cells through inducing BRCC3 expression.

In summary, this study demonstrated that B7-H3 can reduce the resistance to 5-Fu by mediating DNA damage repair via BRCC3 (Fig. 5). These results suggest that new CRC treatments could target B7-H3 overexpression or associated signaling pathways in tumors as a novel approach to antagonize drug resistance.

Acknowledgements

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