BRCA1 Activates a G_2-M Cell Cycle Checkpoint following 6-Thioguanine–Induced DNA Mismatch Damage

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Abstract

Human DNA mismatch repair (MMR) is involved in the response to certain chemotherapy drugs, including 6-thioguanine (6-TG). Consistently, MMR-deficient human tumor cells show resistance to 6-TG damage as manifested by a reduced G_2-M arrest and decreased apoptosis. In this study, we investigate the role of the BRCA1 protein in modulating a 6-TG–induced MMR damage response, using an isogenic human breast cancer cell line model, including a BRCA1 mutated cell line (HCC1937) and its transfectant with a wild-type BRCA1 cDNA. The MMR proteins MSH2, MSH6, MLH1, and PMS2 are similarly detected in both cell lines. BRCA1-mutant cells are more resistant to 6-TG than BRCA1-positive cells in a clonogenic survival assay and show reduced apoptosis. Additionally, the mutated BRCA1 results in an almost complete loss of a G_2-M cell cycle checkpoint response induced by 6-TG. Transfection of single specific small interfering RNAs (siRNA) against MSH2, MLH1, ATR, and Chk1 in BRCA1-positive cells markedly reduces the BRCA1-dependent G_2-M checkpoint response. Interestingly, ATR and Chk1 siRNA transfection in BRCA1-positive cells shows similar levels of 6-TG cytotoxicity as the control transfectant, whereas MSH2 and MLH1 siRNA transfectants show 6-TG resistance as expected. DNA MMR processing, as measured by the number of 6-TG–induced DNA strand breaks using an alkaline comet assay (±z-VAD-fmk cotreatment) and by levels of iododeoxyuridine-DNA incorporation, is independent of BRCA1, suggesting the involvement of BRCA1 in the G_2-M checkpoint response to 6-TG but not in the subsequent excision processing of 6-TG mispairs by MMR. [Cancer Res 2007;67(13):6286–92]

Introduction

Genetic instability plays a central role in the initiation and progression of cancer, causing mutations of tumor suppressor genes and proto-oncogenes. Activation of cell cycle checkpoints is a major mechanism in preventing genetic instability (for reviews, see refs. 1, 2). For example, following DNA double-strand breaks, the G_2-M cell cycle checkpoint can be activated by several proteins called checkpoint Rad proteins, including ataxia-telangiectasia mutated (ATM) and ATM and Rad3-related (ATR), which subsequently activate Chk1 and Chk2 checkpoint kinases, respectively (3, 4). One of the final consequences is considered to be inactivation of Cdc2 kinase and a cell cycle arrest at the G_2 phase. This checkpoint response is felt to be essential to inhibit transfer of damaged genetic information to daughter cells, supporting genetic stability and prevention of cancer.

The *BRCA1* gene is a tumor suppressor gene, which can be mutated in familial breast and ovarian cancers (5). Its product has two BRCT (*BR*CA1 *c*arboxyl *t*erminus) motifs, which are also found in several proteins involved in cell cycle checkpoints and DNA repair (6, 7). Some BRCT-containing proteins, including BRCA1, TopBP1, MDC1, 53BP1, and NBS1, are reported to activate ATR or ATM kinase (8–11), which are critical for subsequent Chk1 or Chk2 kinase activation, respectively. The involvement of BRCA1 in this checkpoint pathway could directly link genomic instability of familial breast/ovarian cancer to a malfunction of mutated BRCA1.

Recently, it was shown that the ATR-Chk1 pathway can be activated after DNA mismatch damage (12–15). Some of these reports indicate that treatment of cells with DNA mismatch–inducing agents including Sn-1 methylating drugs and 6-thioguanine (6-TG) results in phosphorylation of Chk1/Chk2, indicating the activation of ATR-Chk1 and ATM-Chk2 pathways. The ATR-Chk1 pathway plays a central role in the activation of a G_2-M checkpoint by these mismatch repair (MMR)–inducing agents.

In this study, we investigate the function of the BRCA1 protein on DNA mismatch–induced cell cycle regulation. A mutation of BRCA1 results in a strong defect (~100%) in G_2-M cell cycle checkpoint activation by 6-TG, whereas, as we previously reported (9), the mutation results in only a partial defect (~20%) in G_2-M checkpoint activation following ionizing radiation (10 Gy). Our results suggest that the BRCA1 mutation can result in genomic instability related to a deficiency of the G_2-M checkpoint activated by DNA mismatches, possibly leading to point mutations of other tumor suppressor genes or proto-oncogenes.

Materials and Methods

Cell culture. HCC1937 cells were established from a primary breast carcinoma (16) and are homozygous for a BRCA1 mutation, which includes a base insertion at codon 1755, corresponding to a site between the two BRCT repeats (17). HCC1937 cells were transfected with a Myc epitope–tagged wild-type *BRCA1* gene on a vector derived from pcDNA3 (Invitrogen), as previously described (9). The total population of neomycin-resistant cells was used and termed HCC1937 + wtBRCA1. Cells were grown in RPMI 1640 supplemented with 10% FCS in a humidified atmosphere of 10% CO_2 and 90% air at 37°C. 6-TG, which was purchased from Sigma, was dissolved in 0.1 NaOH, diluted to 1.5 mmol/L 6-TG, 20 mmol/L NaOH, and stocked in −20°C. 6-TG was added directly to the medium as specified in each experiment. HCT116 human colorectal cancer cells transfected with the wild-type *MLH1* gene or the empty vector were also used (18). This isogenic pair of HCT116 cell lines was kindly provided by Dr. Francoise Praz (Centre National de la Recherche Scientifique, Villejuif, France). Similar growth conditions were used for the HCT116 cells as described above. The cell population doubling time for both of the

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HCC1937 cell lines was 40 ± 3 h, whereas the cell population doubling time for the HCT116 cell lines was 24 ± 2 h.

Transfection of small interfering RNA. The small interfering RNAs (siRNA) used were 5′-GCUCAGAUCAAUACGGAGAdTdT (a negative control), 5′-GCGUGCCGUAGACUGUCCAdTdT (Chk1), 5′-UCUGCAGAGUGUUG-UGCUUdTdT (MSH2), 5′-GCCAUGUGGCUCAUGUUACdTdT (MLH1), and 5′-GCCAAGACAAAUUCUGUGUdTdT (ATR; ref. 14). All siRNAs were obtained from Dharmacon. All were annealed with the complementary strand with dTdT overhangs. Transfection was done with Oligofectamine as recommended by the manufacturer (Invitrogen). Transfection was done for 30 h and cells were then washed and trypsinized. The cells from one well per 24-well plate were divided into two to three wells and RPMI 1640 was added to the culture.

Western blotting. Total cell extracts were prepared by trichloroacetic acid precipitation to detect all proteins. After extraction of trichloroacetic acid with acetone, samples were dissolved in a loading buffer, and DNA in samples was sheared by sonication before loading onto gels. The following antibodies against the indicated human proteins were used for Western blotting: MSH2 (Ab-2), BRCA1 (Ab-1, MS110; Oncogene Research Products); PMS2 (BD PharMingen); MSH6 and MLH1 (BD Transduction Laboratories); β-actin (Sigma); phosphorylated Cdc2 and phosphorylated Chk1 and Chk2 (Cell Signaling Technology); histone H3, Chk1, Chk2, and Cdc2 (Santa Cruz Biotech); ATR (PA1-450, Alexon Biopharma Research); and phosphorylated histone H3 (Upstate).

Flow cytometry. Cells were fixed with 90% ethanol at −20°C for 1 to 6 days, incubated with RNase, stained with propidium iodide, and then subjected to flow cytometry (Coulter, Epics XL-MCL).

G_2-M checkpoint assay. Cells were treated with 6-TG continuously for up to 4 days. When nocodazole (0.3 μg/mL; Sigma) was added to the medium, the cell density was approximately <60% confluent, for reproducible data. After nocodazole treatment for 18 h, attached and floating cells were collected and subjected to Western blotting as described above.

Confocal microscopy. Cells were treated as indicated above and were then fixed with paraformaldehyde and stained with an anti-BRCA1 antibody (Calbiochem). Samples were washed, stained with secondary antibodies (Alexa Fluor 488 antimouse immunoglobulin G, Molecular Probes), and then subjected to confocal microscopy as described (19, 20).

Comet assay. Cells were treated with 0 or 3 μmol/L 6-TG and with 0 or 20 μmol/L z-VAD-fmk (Biomol) for 3 days, and then subjected to the alkaline comet assay (single-cell gel electrophoresis). The alkaline comet assay was done as described (21). The data were analyzed using >60 comet images captured by Cometscore (version 1.5, TriTek Corp.).

Incorporation of iododeoxyuridine. Cells were incubated with 5 μmol/L iododeoxyuridine (IUdR; Sigma) for 1 cell population doubling time (24 h for HCT116 cells and 40 h for HCC1937 cells) in RPMI 1640 supplemented with 10% dialyzed FCS in 10% CO_2 and 90% air at 37°C. The incorporated nucleosides were analyzed by high-performance liquid chromatography as described (21).

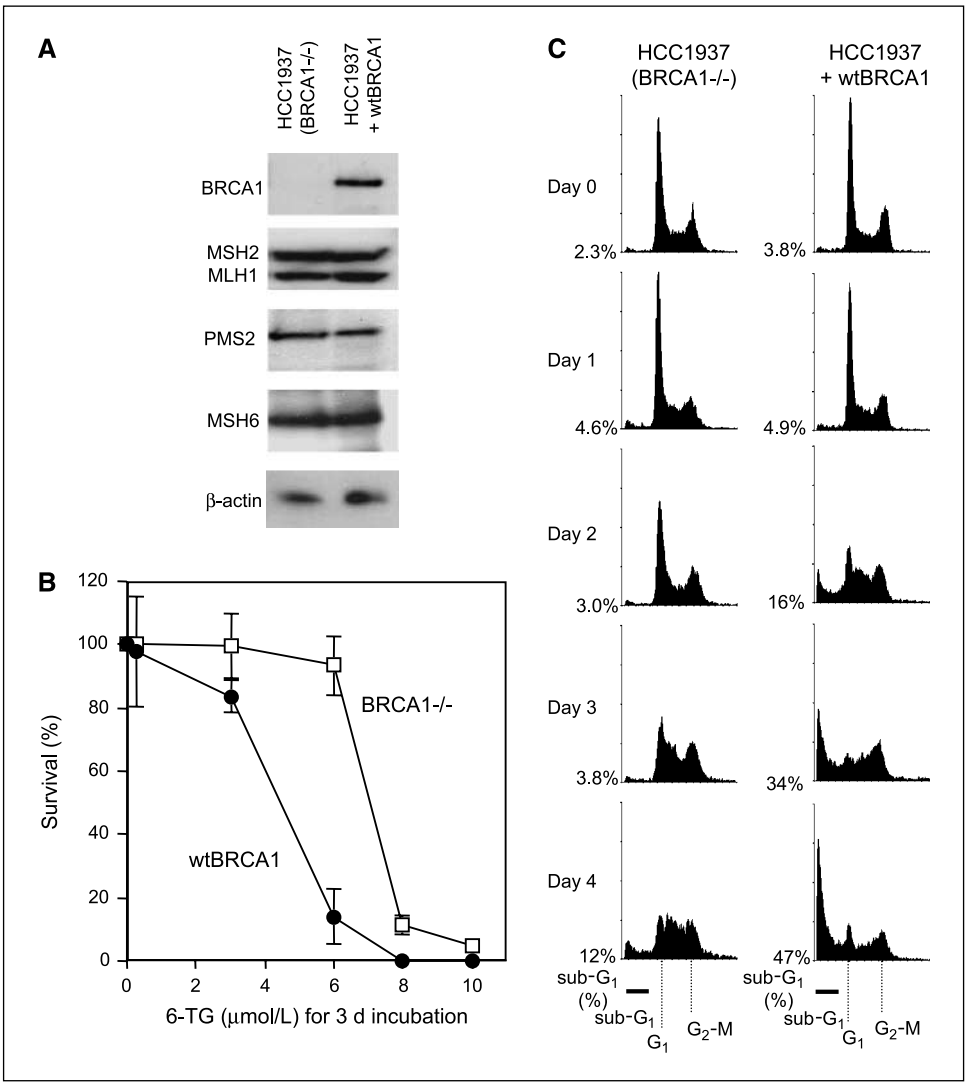

Figure 1. BRCA1 deficiency confers resistance to 6-TG. *A,* HCC1937 with mutated BRCA1 and its transfectant with a wild-type BRCA1 cDNA (HCC1937 + wtBRCA1) were subjected to Western blotting with antibodies against BRCA1, MSH2, MLH1, PMS2, and MSH6. *B,* HCC1937 (*BRCA1*−/−) and HCC1937 + wtBRCA1 (*wtBRCA1*) cells were incubated with 6-TG at the indicated concentrations for 3 d. The cells were washed and cultured in the medium. Colonies were fixed with 0.25% formaldehyde and then stained with 0.5% crystal violet. *C,* both cell populations are continuously treated with 3 μmol/L 6-TG for the indicated days, fixed, stained with propidium iodide, and analyzed by flow cytometry.

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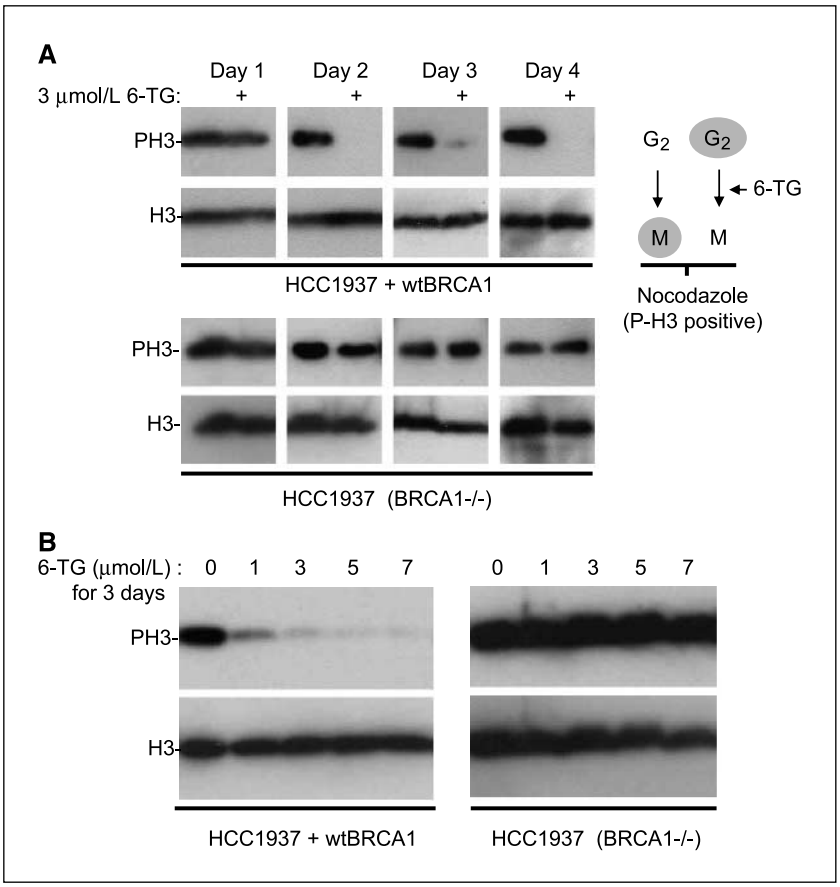

Figure 2. BRCA1 is required for the G_2-M checkpoint activated by 6-TG. *A,* HCC1937 BRCA1-mutant and BRCA1-positive cells were treated with 3 μmol/L 6-TG for the indicated days and then incubated with medium containing 0.3 μg/mL nocodazole for 18 h without 6-TG. The cells were collected and subjected to Western blotting with antibodies against the phosphorylated Ser^{10} of histone H3 (*P-H3*) or against total anti–histone H3 (*H3*). *B,* the cells are treated with the indicated concentrations of 6-TG for 3 d and then incubated with the medium containing 0.3 μg/mL nocodazole for 18 h.

Results

BRCA1 deficiency confers resistance to 6-TG. We used HCC1937, a human breast cancer cell line with mutated BRCA1, and its transfectants with a wild-type BRCA1 cDNA (HCC1937 + wtBRCA1) as our principal cell model (9). Western blotting showed similar levels of expression of the key MMR proteins, MSH2 and MSH6 (MutSα), as well as MLH1 and PMS2 (MutLα), in both cell lines (Fig. 1*A*).

MMR-proficient cells have been reported to be more sensitive to 6-TG, a DNA mismatch–inducing drug, than MMR-deficient cells as the MMR system abortively tries to remove 6-TG–containing bases in the parent strand resulting in DNA nicks in the daughter strand (22). In this study, we examined sensitivity to 6-TG in the genetically matched BRCA1-positive and BRCA1-defective human tumor cells. These cells were incubated with increasing concentrations of 6-TG (up to 10 μmol/L) for 3 days and assessed by colony formation. BRCA1-positive cells are generally more sensitive to 6-TG than BRCA1-mutant cells, with <1% survival after 8 and 10 μmol/L 6-TG treatment (Fig. 1*B*). These results indicate that BRCA1 can be one of determinants of sensitivity to 6-TG.

When these cells are continuously treated with 3 μmol/L 6-TG, which induces futile cycles of DNA MMR (22), both cell lines showed changes in their cell cycle profiles (Fig. 1*C*). At days 2 to 4, BRCA1-positive cells have larger sub-G_1 populations, suggesting induction of apoptosis by 6-TG.

BRCA1 is required for the G_2-M checkpoint activated by 6-TG. Because a continuous exposure to 6-TG can arrest cells in G_1, S, and G_2-M phases (14, 23), possibly due to inhibition of DNA synthesis, it is technically difficult to distinguish a G_2-M checkpoint response by flow cytometry using propidium iodide staining. To examine the G_2-M checkpoint response, we used an antibody against the phosphorylated Ser^{10} of histone H3 as a mitotic marker (9). Following 0 or 3 μmol/L 6-TG for 1 to 4 days, nocodazole was added to arrest cells in M phase to quantitate progression of G_2 cells into mitosis. After 6-TG treatment for 2 to 4 days, the band of phosphorylated histone H3 was strongly reduced in BRCA1-positive cells (Fig. 2*A*), suggesting that a G_2-M checkpoint was activated to inhibit cells from entering into M phase. A quantitative dilution/exposure of the band signals indicated a 20-fold inhibition by day 3 of 6-TG treatment (data not shown). In contrast, 6-TG treatment of BRCA1-mutant cells did not activate the G_2-M checkpoint as these cells progress into mitosis (Fig. 2*A*). The checkpoint response was dependent on the concentration of 6-TG in the BRCA1-positive cells (Fig. 2*B*). These results indicate that BRCA1 is required for the G_2-M checkpoint activation by 6-TG in a dose-dependent manner.

6-TG treatment activates Chk1 in BRCA1-positive cells without nuclear focus formation. We have previously shown that Chk1 and ATR are required for the G_2-M checkpoint induced by 6-TG (14, 24). In this study, we used an antibody against the phosphorylated Ser^{345} of Chk1 to examine the involvement of the checkpoint kinase Chk1 in these BRCA1-positive and BRCA1-mutant cell lines. Phosphorylation of Ser^{345} is essential for the full activation of Chk1 (25). The phosphorylation of Chk1 at Ser^{345} is strongly induced in the BRCA1-positive cells at days 2 to 4 (Fig. 3*A*) but not in the BRCA1-mutant cells. On the other hand, there is a

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small delay in the phosphorylation of Chk1 at Ser^{317} and Chk2 at Thr^{68} in the BRCA1-mutant cells. Phosphorylated Tyr^{15} Cdc2 is also reduced in BRCA1-mutant cells. The total protein levels of Chk1 and Cdc2 are changed during incubation with 6-TG, whereas histone H3 and β-actin levels remain constant. These results suggest that phosphorylation of Chk1 at Ser^{345} is critical in the BRCA1-dependent G_2-M checkpoint response.

Ionizing radiation can induce nuclear foci of BRCA1 (26). Therefore, we examined whether DNA mismatches after 6-TG treatment can also induce BRCA1 foci. Incubation of 6-TG did not increase BRCA1 foci during the 4-day exposure (Fig. 3*B*, *D0–D4*), whereas ionizing radiation (10 Gy) resulted in ionizing radiation–induced foci in 83% of BRCA1-transfected cells when assessed at 6 h after ionizing radiation treatment. Additionally, nuclear foci of MLH1 were not formed in BRCA1-positive cells after 6-TG treatment (data not shown). These results suggest that the macro repair processing after 6-TG damage differs from that after ionizing radiation damage in these BRCA1-positive cells.

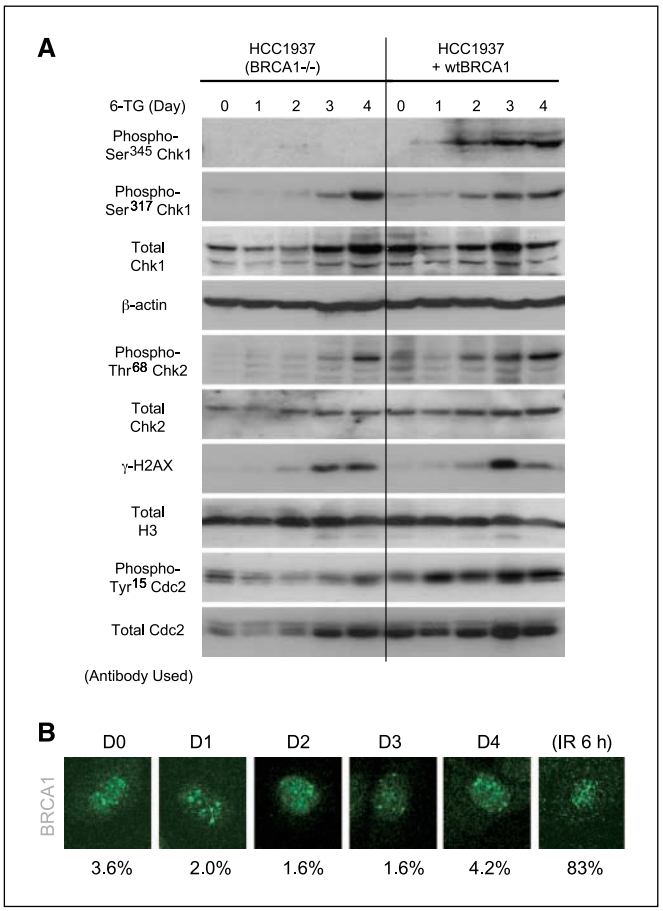

Figure 3. Phosphorylation of Chk1 Ser^{345} is reduced in BRCA1-mutant cells following 6-TG treatment. *A,* HCC1937 BRCA1-mutant and BRCA1-positive cells were treated with 3 μmol/L 6-TG for the indicated days. Total cell lysates were subjected to Western blotting with the indicated antibodies. The protein levels of β-actin and histone H3 were used as two loading controls. *B,* HCC1937 BRCA1-positive cells were treated with 3 μmol/L 6-TG for the indicated days or with ionizing radiation (10 Gy), then fixed and stained with anti-BRCA1 antibody. Samples were then analyzed by confocal microscopy. The indicated percentages are nuclear focus–forming cells per total cells (>50 cells). 6-TG–treated cells were analyzed daily for 4 d (*D1–D4*) and compared with untreated cells [day 0 (*D0*)]. Ionizing radiation–treated cells were analyzed 6 h following 10 Gy (*IR 6 hr*).

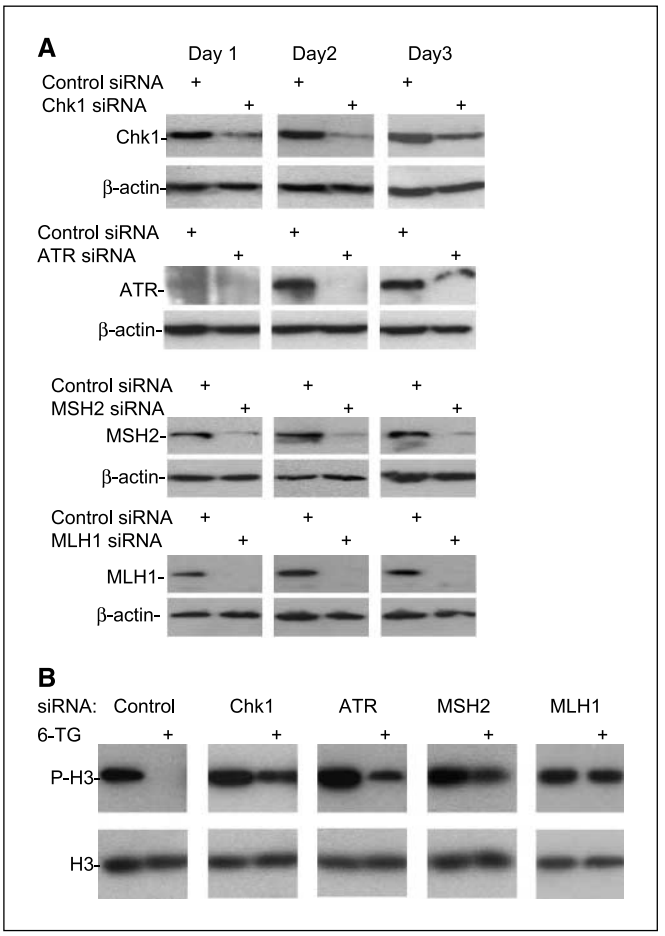

Figure 4. MSH2, MLH1, Chk1, and ATR are required for the BRCA1-dependent G_2-M checkpoint following 6-TG treatment. *A,* the indicated siRNAs were transfected into HCC1937 BRCA1-positive cells. After transfection, cells were incubated in complete medium without 6-TG for the indicated times, harvested, and analyzed by Western blotting with the indicated antibodies. *B,* HCC1937 BRCA1-positive cells were transfected with siRNA and then treated with 0 or 4.5 μmol/L 6-TG for 2 d and finally incubated in medium containing 0.3 μg/mL nocodazole for 7 h before harvesting time. Cells were collected and subjected to Western blotting with antibodies against the phosphorylated Ser^{10} of histone H3 and against total anti–histone H3.

MSH2, MLH1, ATR, and Chk1 are required for the BRCA1-dependent G_2-M checkpoint. We have shown that Chk1 and ATR regulate the G_2-M checkpoint induced by 6-TG in MMR-proficient cells (14, 24). In this study, we questioned whether specific siRNA knockdown of proteins (MSH2, MLH1, ATR, or Chk1) in BRCA1-positive cells would affect the observed 6-TG–induced G_2-M checkpoint response. Using these four siRNAs, a clear inhibition of the targeted proteins was observed (Fig. 4*A*), with ATR and Chk1 showing some recovery of protein expression by day 3. The ATR siRNA also inhibited an ATR protein level at days 2 and 3 whereas, at day 1, the protein level was also low in the control siRNA.

After treatment with 4.5 μmol/L 6-TG for 2 days, the G_2-M checkpoint could be strongly activated in BRCA1-positive cells transfected with the control siRNA because there is no band of phosphorylated histone H3 in the 6-TG–treated lane (Fig. 5*B*). In contrast, inhibition of the individual protein levels of Chk1, ATR, MSH2, and MLH1 reduced the G_2-M checkpoint response, indicating that the BRCA1-dependent checkpoint activation may require all four proteins.

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siRNA-mediated reduction of MSH2 and MLH1 but not ATR and Chk1 can confer resistance to 6-TG. Colony formation assays after 6-TG treatment for 3 days in BRCA1-positive cells were done following single siRNA transfection against MSH2, MLH1, ATR, and Chk1, respectively. As expected, siRNA reduction of MSH2 and MLH1 results in resistance to 6-TG in the BRCA1-positive cells (Fig. 5*A*). However, the partial reduction of ATR and Chk1 protein expression in BRCA1-positive cells showed similar cytotoxicity to control siRNA–transfected cells (Fig. 5*B*), suggesting that the ATR-Chk1 pathway is principally involved in the G_2-M cell cycle checkpoint activation but not in determining clonogenic cell survival after 6-TG treatment in these cells. However, we found that clonogenic cell survival in BRCA1-positive cells after siRNA transfection with either ATR or Chk1 without 6-TG treatment was significantly lower (>50%) than after siRNA transfection with MSH2 or MLH1 (see legend of Fig. 5). These results of reduced clonogenic survival after siRNA transfection of ATR and Chk1 support prior studies showing that ATR and Chk1 are important for overall cell viability (3, 27) but not for determining 6-TG cytotoxicity in this HCC1937 cell model.

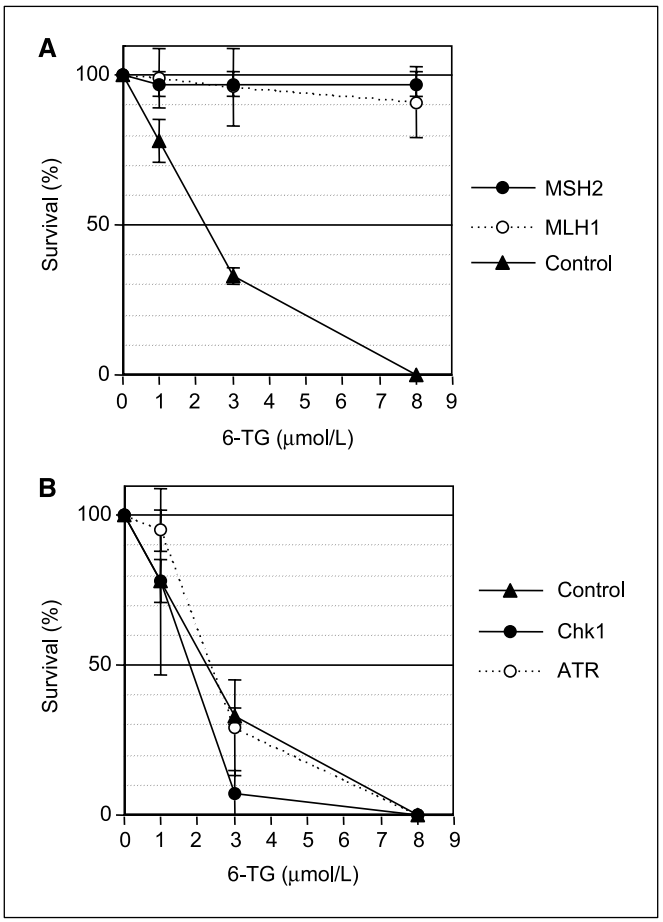

Figure 5. siRNA knockdown of MSH2 and MLH1 but not ATR and Chk1 confers resistance to 6-TG in BRCA1-positive cells. *A* and *B,* the siRNAs against MSH2, MLH1, Chk1, and ATR were transfected to the HCC1937 BRCA1-positive cells. Cells were treated with different concentrations of 6-TG for 3 d, washed, and cultured in the medium. After ~3 wk, cells were stained and colonies (>50 cells) counted. The cell survival ratios (± SD) without 6-TG were 100% (control), 85 ± 22% (MSH2 siRNA), 94 ± 8% (MLH1 siRNA), 39 ± 12% (Chk1 siRNA), and 43 ± 23% (ATR siRNA).

BRCA1 is not involved in subsequent MMR processing of chemically induced DNA mismatches by 6-TG and IUdR. Alkaline comet assays were done to examine DNA breaks induced by 6-TG, including single-strand breaks because we have previously shown that single-strand breaks are intermediate products of MMR processing of 6-TG DNA mismatches (28). After 3 μmol/L 6-TG treatment for 3 days, similar comet tails were observed in both BRCA1-mutant and BRCA1-positive cells (Fig. 6*A*). Similar tails were also observed in both BRCA1-mutant and BRCA1-positive cells after cotreatment with 6-TG and z-VAD-fmk for 3 days, where z-VAD-fmk was used to inhibit double-strand breaks induced by apoptosis. Quantification of the images indicates that both BRCA1-mutant and BRCA1-positive cells have similar amounts of DNA damage (percent DNA in tail) after 6-TG treatment (Fig. 6*B*).

To further investigate the involvement of BRCA1 in DNA MMR processing independent of the G_2-M checkpoint response, we used an incorporation assay of the nucleoside analogue IUdR (Fig. 6*C*). We have previously shown that the IUdR incorporation into DNA (principally as G:IU mispairs) is efficiently processed by MMR without enhanced cytotoxicity in MMR-positive human cancer cells, using low concentrations (1–10 μmol/L) of IUdR (29–31). As expected, HCT116 human colon cancer cells with wild-type MLH1 proteins showed reduced incorporation of IUdR following 5 μmol/L IUdR for 1 cell population doubling (24 h). However, BRCA1-mutant and BRCA1-positive cell lines did not show a significant difference in IUdR-DNA incorporation following similar IUdR exposures, suggesting that BRCA1 is not involved in MMR processing of these types of chemically induced DNA mismatches. Whereas the overall levels of IUdR-DNA incorporation are actually higher in the HCC1937 cell lines compared with HCT116 cell lines, these overall differences reflect intracellular metabolism of IUdR, principally by thymidine kinase, before IUdR-DNA incorporation as we previously published (29, 30). Technically, we could not determine the levels of 6-TG-DNA incorporation by standard high-performance liquid chromatography methods as used in measuring IUdR-DNA incorporation, in either the HCT116 or HCC1937 cell models, because levels are reported to be very low (<1%) and intracellular purine analogue drug metabolism is complex (32).

Discussion

In this study, BRCA1 is shown to be involved in a G_2-M checkpoint induced by 6-TG, a DNA mismatch inducing drug (Fig. 2). The contribution of BRCA1 to this checkpoint response is quite strong because the BRCA1-positive cells have >95% inhibition of M-phase entry whereas the mutant cells have almost 0% inhibition after 3 μmol/L 6-TG treatment for 2 to 4 days. 6-TG treatment in BRCA1-mutant cells also fails to phosphorylate Chk1 at Ser^{345} (Fig. 3). A siRNA-mediated reduction of protein levels of MSH2, MLH1, ATR, and Chk1 in BRCA1-positive cells markedly reduces the G_2-M checkpoint response (Fig. 4), suggesting that the BRCA1-dependent checkpoint activation requires two MMR proteins, MSH2 and MLH1, and two checkpoint proteins, Chk1 and ATR. These results suggest a novel mechanism leading to genomic instability of BRCA1-defective cells.

Because a MMR complex (MutSα/MutLα) containing both MSH2 and MLH1 proteins is the sensor of DNA mismatches (33), this complex will most likely be located upstream in our proposed signaling pathway. Additionally, BRCA1 can interact with MLH1 (34); BRCA1 may be downstream of the MMR complex. BRCA1 is

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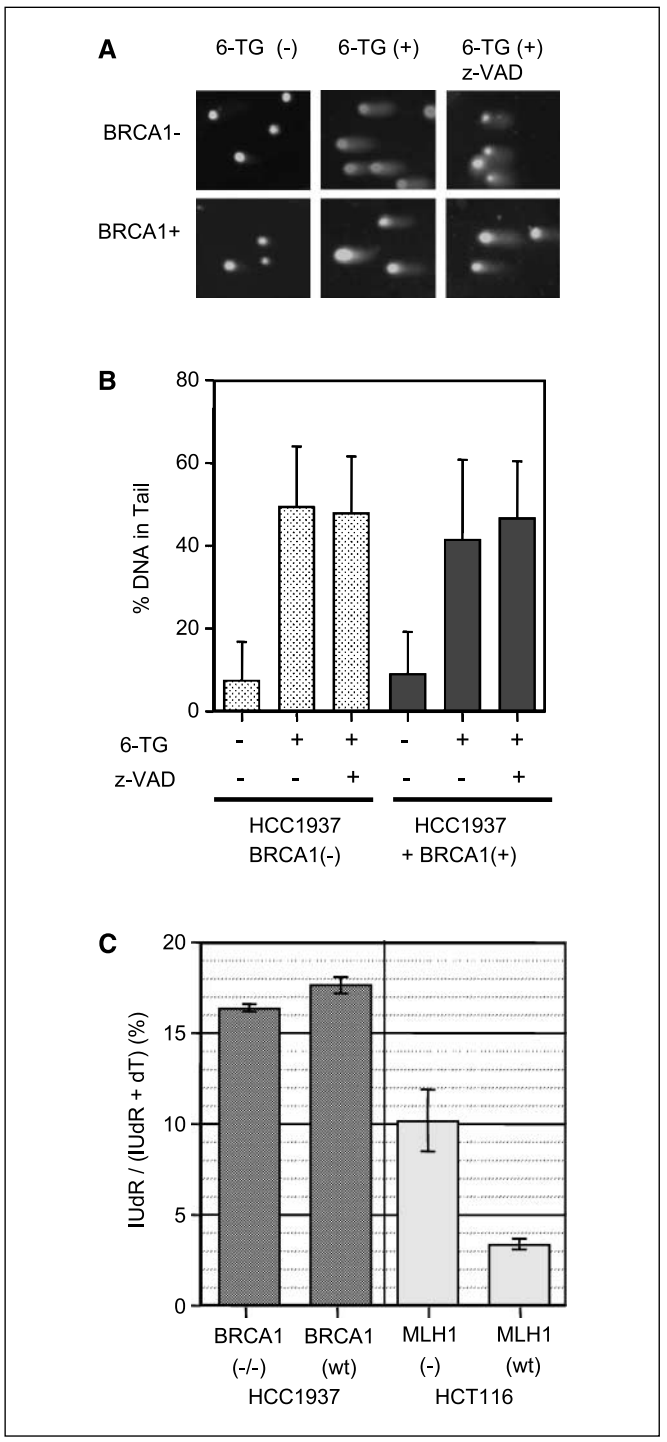

Figure 6. BRCA1 is not essential for subsequent MMR processing of 6-TG or IUdR DNA mismatches. *A* and *B,* HCC1937 BRCA1-positive cells were incubated with 3 μmol/L 6-TG with or without 20 μmol/L z-VAD-fmk(*z-VAD*) for 3 d, and the alkaline comet assay was done to quantitate DNA breaks using Cometscore (version 1.5, TriTek). z-VAD-fmk is used to inhibit 6-TG–induced double-strand breaks mediated through apoptosis. *C,* HCC1937 BRCA1-mutant and BRCA1-positive cells were exposed to 5 μmol/L IUdR for 1 population doubling (40 h) and percentage of IUdR DNA replacement of thymidine (*dT*) was quantitated by high-performance liquid chromatography. For comparison, MMR^- (HCT116, $MLH1^-$) and MMR^+ (HCT116 + a wild-type *MLH1*$^+$ cDNA) human colon cancer cells were similarly treated with 5 μmol/L IUdR for 1 population doubling (24 h). Note that the total IUdR-DNA levels are different between the two cell models (HCC1937 versus HCT116) and cannot be directly compared.

recognized to be a checkpoint Rad protein in responses to ionizing radiation damage, leading to activation of ATR (9) as well as other checkpoint Rad proteins. Our data indicate that 6-TG treatment also induces Chk1 Ser^{345} phosphorylation in a BRCA1-dependent manner (Fig. 3*A*). Because activated ATR can phosphorylate Chk1 directly (2, 27), a direct MSH2/MLH1-BRCA1-ATR-Chk1 signaling pathway is possible. Alternatively, 6-TG–induced signal transmission from MSH2/MLH1 to BRCA1 may be indirect via the creation of DNA single-strand breaks, as we previously suggested (14, 28). We also found that Chk2 can be phosphorylated in response to 6-TG treatment (Fig. 3; refs. 14, 15), suggesting an additional signaling pathway, although the significance of this pathway is unknown at this time.

We have found that DNA MMR–proficient human tumor and murine cells are significantly more sensitive (~10–100-fold) to low concentrations (1–3 μmol/L) of 6-TG than MMR-deficient cells (28–30), as the MMR system abortively tries to remove 6-TG–containing bases in a futile cycle (28). BRCA1-positive cells are also more sensitive to higher concentrations of 6-TG than BRCA1-mutant cells (~10-fold after 6 μmol/L × 3 days; Fig. 1), suggesting that the G_2-M checkpoint abrogation may be one of factors affecting cell survival after 6-TG treatment. It follows that if BRCA1 is mutated in an otherwise intact MMR-competent cell, then the damage signaling to the G_2 checkpoint would reduce removal of the 6-TG mispairs and reduce apoptosis, providing a survival advantage to these BRCA1-mutant cells. Consistently, BRCA1-dependent sensitivity to 6-TG was reversed by reduction of MSH2 and MLH1 protein levels in BRCA1-positive cells (Fig. 5*A*). Interestingly, ATR and Chk1 siRNA transfection did not confer resistance to 6-TG, indicating that the observed partial reduction of ATR and Chk1 protein expression (Fig. 4*A*) does not affect 6-TG cytotoxicity in this cell model (Fig. 5*B*). However, the partial inhibition of ATR or Chk1 by siRNA transfection alone did result in a >50% lower cell survival without 6-TG treatment compared with MSH2 or MLH1 siRNA transfection in these BRCA1-positive cells (Fig. 5). The cytotoxic effect of the ATR or Chk1 knockdowns may mutually compensate for the cytotoxic effect of 6-TG. Therefore, our data showing no clonogenic survival variation in $BRCA1^+$ transfectants under these specific ATR or Chk1 siRNA transfection conditions may reflect the complexity of this experimental design. We attempted to use a BRCA1 siRNA transfection in HCT116 + MLH1 cDNA cells but were unable to effectively (>80%) reduce BRCA1 protein expression for a 3-day interval to assess 6-TG cytotoxicity (data not shown).

DNA mismatch excision was measured by IUdR incorporation (Fig. 6*C*), as we have shown that G:IU mismatches are efficiently recognized and removed by MMR (29–31). However, IUdR-DNA incorporation repair was independent of the BRCA1 protein in this cell model. The alkaline comet assay (Fig. 6*A* and *B*) also indicates that BRCA1 may not contribute to excision processing of 6-TG damage, including the creation of single-strand DNA breaks through a proposed futile cycle. These results suggest that BRCA1 may not be directly involved in mismatch damage processing per se but is involved in initial G_2-M checkpoint response induced by MMR. However, we were not able to detect levels of 6-TG DNA incorporation in the cell models used, similar to other studies (32), and cannot provide this direct evidence as supporting our hypothesis.

Interestingly, there was a small difference in phosphorylation of Chk1 Ser^{317} between BRCA1-mutant and BRCA1-positive cells, whereas phosphorylation of Chk1 Ser^{345} is markedly reduced in

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BRCA1-mutant cells (Fig. 3). Our results suggest that phosphorylation of Chk1 Ser^{345} (together with Ser^{317}) is critical for activation of a 6-TG–induced G_2-M checkpoint. Additionally, there were small differences in phosphorylation time courses of Chk2 and H2AX between BRCA1-mutant and BRCA1-positive cells (Fig. 3), suggesting that Chk2 and H2AX are not major factors regulating the G_2-M checkpoint, consistent with other published reports (35, 36). Chk2 can be phosphorylated by ATM, whereas H2AX can be phosphorylated by both ATM and ATR (37, 38). Our results suggest that BRCA1 is not a major factor in ATM activation.

In summary, this is the first report to suggest that BRCA1 may be involved in the G_2-M cell cycle checkpoint following DNA mismatch damage by 6-TG. Taken together with our previously published data (14, 15, 28), we suggest that 6-TG–induced DNA mismatches can activate an MSH2/MLH1-BRCA1-ATR-Chk1 pathway, leading to a G_2 arrest. However, this proposed BRCA1 signaling pathway may not be directly involved in the subsequent MMR processing of chemically induced DNA mismatches. These differences in the MMR responses to 6-TG damage underscore the complexity of MMR as recently reviewed (33).

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BRCA1 Activates a G_2-M Cell Cycle Checkpoint following 6-Thioguanine–Induced DNA Mismatch Damage

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