Fanconi anemia D2 protein confers chemoresistance in response to the anticancer agent, irofulven

Yutian Wang,1 Timothy Wiltshire,2 Jamie Senft,1 Sharon L. Wenger,3 Eddie Reed,1,2 and Weixin Wang1,2

1Mary Babb Randolph Cancer Center, 2Department of Microbiology, Immunology, and Cell Biology, and 3Department of Pathology, West Virginia University School of Medicine, Morgantown, West Virginia

Abstract
The Fanconi anemia-BRCA pathway of genes are frequently mutated or epigenetically repressed in human cancer. The proteins of this pathway play pivotal roles in DNA damage signaling and repair. Irofulven is one of a new class of anticancer agents that are analogues of mushroom-derived illudin toxins. Preclinical studies and clinical trials have shown that irofulven is effective against several tumor cell types. The exact nature of irofulven-induced DNA damage is not completely understood. Previously, we have shown that irofulven activates ATM and its targets, NBS1, SMC1, CHK2, and p53. In this study, we hypothesize that irofulven induces DNA double-strand breaks and FANCD2 may play an important role in modulating cellular responses and chemosensitivity in response to irofulven treatment. By using cells that are proficient or deficient for FANCD2, ATR, or ATM, we showed that irofulven induces FANCD2 monoubiquitination and nuclear foci formation. ATR is important in mediating irofulven-induced FANCD2 monoubiquitination. Furthermore, we showed that FANCD2 plays a critical role in maintaining chromosome integrity and modulating chemosensitivity in response to irofulven-induced DNA damage. Therefore, this study suggests that it might be clinically significant to target irofulven therapy to cancers defective for proteins of the Fanconi anemia-BRCA pathway. [Mol Cancer Ther 2006;5(12):3153–61]

Received 7/20/06; revised 9/28/06; accepted 11/1/06.

Grant support: National Cancer Institute grant 5R03CA107979 (W. Wang).

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Note: Current address for E. Reed: Centers for Disease Control and Prevention, Atlanta, Georgia.

Requests for reprints: Weixin Wang, Mary Babb Randolph Cancer Center, West Virginia University, 1835 Health Sciences South, P.O. Box 9300, Morgantown, WV 26506. Phone: 304-293-2243; Fax: 304-293-4667. E-mail: wswang@hsc.wvu.edu

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Introduction
Fanconi anemia is a genetic cancer-susceptibility syndrome characterized by congenital abnormalities, bone marrow failure, and cellular sensitivity to DNA cross-linking agents (1, 2). There are at least 12 Fanconi anemia complementation groups (A, B, C, D1, D2, E, F, G, I, J, L, and M), and 10 Fanconi anemia genes (A, C, D1/BRCA2, D2, E, F, G, J, L, and M) have been cloned (1–8). All of the Fanconi anemia proteins function in a common pathway. Fanconi anemia proteins (A, B, C, E, F, G, L, and M) assemble in a nuclear complex that is required for monoubiquitination/activation of the downstream FANCD2 protein (1, 2, 4, 6, 9–13). FANCL has been shown to possess the E3 ubiquitin ligase activity to monoubiquitinate FANCD2 at Lys561 (11). The deubiquitinating enzyme of FANCD2, USP1, has also been recently identified (14). FANCD1 was shown to be identical to BRCA2 (15). FANCD2 colocalizes with BRCA1 and BRCA2, and BRCA1 is required for monoubiquitination and foci formation of FANCD2 in response to DNA damage (1, 2, 4, 16–18). Therefore, BRCA1, BRCA2 (FANCD1), and Fanconi anemia proteins functionally merge in a common Fanconi anemia-BRCA pathway of DNA damage response (1, 2).

The Fanconi anemia-BRCA pathway of DNA damage response is frequently impaired in cancers due to genetic mutation or epigenetic repression (1, 2, 4, 9, 10, 19). BRCA1 and BRCA2 (FANCD1) are frequently mutated in familial breast and ovarian cancers (>50%; refs. 1, 20, 21). Germ line mutations in FANCD1 (BRCA2), FANCA, FANCC, or FANCG have been found in pancreatic cancer (>10%; refs. 22–27). The BRCA2 (FANCD1) interacting protein, EMSY, which binds to BRCA2 and represses its function, was found to be amplified in sporadic breast (13%) and high-grade ovarian (17%) cancers (28). FANCF, a protein upstream of FANCD2 and essential for its activation, is silenced by promoter methylation in several types of sporadic cancers including ovarian (21%), breast (17%), head and neck (15%), non–small cell lung (14%), and cervical (30%) cancers (19, 29–31). The silencing of FANCF may be linked to acquired cisplatin resistance in a subset of ovarian cancers (31).

Irofulven (6-hydroxymethylacylfulvene, HMAF; MGI 114, NSC no. 683863) is a member of a new class of anticancer agents that are analogues of mushroom-derived illudin toxins. Preclinical studies and clinical trials have shown that irofulven is effective against several tumor cell types (32–40). Thus far, the structure and nature of DNA damage caused by irofulven have not yet been fully characterized. Earlier studies suggested that the DNA damage caused by the illudin family of compounds might be repaired by the nucleotide excision repair pathway (41, 42). A recent study indicated that...
transcription-coupled nucleotide excision repair is the exclusive pathway in repairing illudin S and irofulven-elicted DNA lesions (43). However, in these studies, the homologous recombination pathway for DNA double-strand break repair was not evaluated (41–43). Previously, we have shown that irofulven activates ATM and its targets, NBS1, SMC1, CHK2, and p53 (44). Recent reports indicate that ATM and CHK2 are specifically activated by drug (calicheamicin) or radiation-induced double-strand breaks (45, 46), and FANCD2 may play a role in homologous recombination pathway of double-strand break repair (1, 2). Therefore, we hypothesize that irofulven induces DNA double-strand breaks, and that FANCD2 may play an important role in maintaining chromosome stability and modulating chemosensitivity in response to irofulven.

To explore whether certain genetic defects in human cancer can be exploited to achieve preferential therapeutic outcomes by irofulven, we did this study to investigate the roles that FANCD2 might play in irofulven-induced DNA damage response. We showed that irofulven induces the monoubiquitination and foci formation of FANCD2. Irofulven-induced FANCD2 monoubiquitination is mediated by ATR. FANCD2 is critical for maintaining chromosome integrity and modulating chemosensitivity in response to irofulven.

Materials and Methods

Cell Culture

All cell lines were maintained in various media supplemented with 10% fetal bovine serum in a 37°C incubator with 5% CO2 atmosphere. Human ovarian cancer cell lines A2780, CAOV3, and OVCAR3 were cultured in RPMI 1640. Human ovarian cancer cell line SKOV3 was cultured in McCoy’s 5A medium. The vector and short hairpin FANCD2 (shFANCD2) stably transfected SKOV3 cells were cultured in McCoy’s 5A medium containing 200 μg/mL of G418 (Invitrogen, Carlsbad, CA). The SKOV3 cells on a 100 mm dish were then infected with 1 mL of viral supernatant and 4 μg/mL of doxycycline (Sigma, St. Louis, MO). The vector and ATM-transfected AT fibroblasts (AT22JEE-T-pEBS7 and AT22JEE-T-pEBS7-YZ5, generously provided by Dr. Yosef Shiloh, Tel Aviv University, Israel; ref. 47) were grown in DMEM with 10% fetal bovine serum in a 37°C incubator and 5% CO2 atmosphere. The SKOV3 cells on a 100 mm dish were then infected with 1 mL of viral supernatant and 4 μg/mL of doxycycline (Invitrogen). For FLAG-tagged kinase-dead ATR induction, cells were treated with 1.5 μg/mL of doxycycline (Sigma) for 48 hours. The Phoenix-ampho packaging cells (American Type Culture Collection, Manassas, VA) were grown in DMEM.

Clonogenic Survival Assay

To determine chemosensitivity and IC50 concentration, the clonogenic survival assay was done on 60 mm cell culture dishes as described previously (44). Cells were treated with different concentrations of irofulven for 1 hour, followed by drug-free incubation for ~10 days. Colonies were stained with crystal violet and counted if 50 or more cells were present. The IC50 concentration was calculated as the irofulven concentration that kills 50% of colonies of untreated controls.

Western Blotting

Western blotting was done as described previously (44). Total cellular extracts (50 μg) were electrophoresed in SDS-PAGE gels. The primary antibodies used included: mouse anti-human actin (1:10,000 dilution), mouse anti-FLAG (1:200 dilution; Sigma), goat anti-human ATR (1:500 dilution), and mouse anti-human FANCD2 (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibodies used were: sheep anti-mouse IgG-HRP (1:2,000 dilution; GE Healthcare Bio-Sciences Corp., Piscataway, NJ) and donkey anti-goat IgG-HRP (1:2,000 dilution; Santa Cruz Biotechnology). The relative amount of FANCD2 protein bands was quantified by densitometry.

Immunofluorescent Staining

Cells were plated on cover-slips and treated with irofulven for 1 hour followed by 12 hours of drug-free incubation. Cells were then fixed and stained with mouse anti-human FANCD2 (1:200 dilution; Santa Cruz Biotechnology). After staining with Alexa-fluor 546-conjugated goat anti-mouse IgG secondary antibody (1:200 dilution; Invitrogen), slides were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) containing 5 ng/mL of 4’,6-diamidino-2-phenylindole. Staining images were captured with an Olympus Provis AX70 fluorescence microscope (Olympus, Melville, NY), and Spot digital camera and software (Diagnostic Instruments, Sterling Height, MI).

RNA Interference

To knock-down ATR, one pair of 65-nucleotide short hairpin ATR oligos containing the target sequence of AACAATGGTATGGTGGTTGA (49), and one pair of 65-nucleotide short hairpin bacterial green fluorescence protein oligos containing the target sequence of TGAGAGCGTTCGACCATAGCA (44) were synthesized. After annealing, the 65-bp double-strand short hairpin ATR and short hairpin GFP fragments were inserted into pSuper.retro.puro vector (OligoEngine, Seattle, WA) and transfected into the Phoenix-ampho packaging cells by calcium phosphate precipitation. The viral supernatants from 100 mm dishes were collected 48 hours after transfection. The SKOV3 cells on a 100 mm dish were then infected with 1 mL of viral supernatant and 4 μL (4 mg/mL) of polybrene (Sigma). The medium was replaced with fresh medium 24 hours after infection. Cells were treated with irofulven 48 hours after infection. To knock-down FANCD2, three pairs of 65-nucleotide...
shFANCD2 oligos containing target sequences of AAGGTTCGCCAGTTGGTGATG, AAGTCAGCTATAGATATGAG, and AAGAAATAAGATTCGATCAGG were designed and synthesized. After annealing, these three 65-bp double-stranded shFANCD2 fragments were inserted into pSilencer 2.1-U6-neo vector (Ambion, Austin, TX) and transfected into SKOV3 cells with Fugene 6 (Roche Applied Science, Indianapolis, IN). The pSilencer 2.1-U6-neo vector containing the scrambled sequence (Ambion) was transfected as the nonspecific control. Stable cell lines were established by selecting in medium containing G418.

Metaphase Spread

Cells were treated with irofulven for 1 h followed by 24 h of drug-free incubation. Colcemid (400 ng/mL; Biosciences, La Jolla, CA) was added to the medium 4 h before harvesting. After trypsinization, cells were washed once with PBS. Cell pellets were resuspended in 75 mmol/L of KCl and incubated at 37°C for 8 min. After centrifugation, cells were fixed using a 3:1 absolute methanol to glacial acetic acid ratio at 4°C for 2 h and then washed twice with fixative. Cells were resuspended in fixative and dropped onto slides. Slides were air-dried at room temperature and stained with 5% Gurr's Giemsa stain (Biomedical Specialties, Santa Monica, CA) for 7 min. Slides were rinsed twice with distilled water and air-dried. The images were recorded by light microscopy using an Olympus Provis AX70 fluorescence microscope (Olympus), and the Spot digital camera and software (Diagnostic Instruments).

Results

FANCD2 Is Monoubiquitinated in Response to Irofulven Treatment

To explore the role that FANCD2 might play in irofulven-induced chemosensitivity, ovarian cancer cell lines A2780, CAOV3, SKOV3, and OVCAR3 were treated with irofulven, FANCD2 ubiquitination status was assessed by Western blot. The results showed that FANCD2 was monoubiquitinated, as indicated by the larger form of FANCD2 (FANCD2-L), in all of the ovarian cancer cell lines after irofulven treatment. Some constitutive FANCD2 monoubiquitination was observed in SKOV3 cells (Fig. 1A).

FANCD2 forms foci at DNA damage sites, which are dependent on monoubiquitination (1, 2, 4, 16–18). Reports have indicated that BRCA1 is required for FANCD2 monoubiquitination and foci formation in response to DNA damage (4, 18, 50). The ovarian cancer cell line SKOV3 is known to harbor a functional BRCA1 (51). To confirm that FANCD2 is monoubiquitinated and activated in response to irofulven-induced DNA damage, SKOV3 cells were treated with irofulven, immunofluorescent staining results showed that FANCD2 forms distinct foci after irofulven treatment (Fig. 1B).

The monoubiquitination of FANCD2 after irofulven treatment was further confirmed in the vector, wild-type FANCD2 and K561R mutant (the monoubiquitination site Lys561 was mutated) FANCD2-transfected FA-D2 fibroblasts (PD20F; ref. 4). Western blot results indicated that increased FANCD2 monoubiquitination was only observed in FANCD2-transfected cells. No monoubiquitinated FANCD2 was found in vector or K561R mutant FANCD2-transfected cells after irofulven treatment (Fig. 1C). Together, these results showed that FANCD2 is monoubiquitinated and activated following irofulven treatment.
FANCD2 Monoubiquitination Is Mediated by ATR

It has been reported that the phosphorylation of FANCD2 is mediated by ATM or ATR in response to IR or psoralen/UV-A (52, 53). The monoubiquitination of FANCD2 requires ATR, but not ATM, in response to IR, hydroxyurea, or mitomycin C (54). Thus far, the structure and nature of DNA damage caused by irofulven have not been fully characterized. We hypothesize that irofulven induces the generation of double-strand breaks based on the finding that irofulven activates ATM and its target genes NBS1, SMCI, and CHK2 (44). We therefore think it is important to determine whether irofulven-induced FANCD2 monoubiquitination is mediated by ATM or ATR. To examine whether ATM was involved in irofulven-induced FANCD2 monoubiquitination, the vector and FLAG-tagged ATM-transfected AT fibroblasts (AT22JE-T-pEB57 and AT22JE-T-pEB57-YZ5; ref. 47) were treated with irofulven. As shown in Fig. 2A, there was little difference in FANCD2 monoubiquitination between untreated cells. The expression of ATM, as indicated by Western blotting with anti-FLAG antibody, did not cause any further increase of FANCD2 monoubiquitination following irofulven treatment. Instead, the ratio of FANCD2-L (monoubiquitinated) to FANCD2-S (nonubiquitinated) was slightly decreased (from 1.5 to 1.1) between the vector and ATM-transfected AT cells following irofulven treatment. This suggests that ATM is not important for irofulven-induced FANCD2 monoubiquitination.

To test whether ATR was involved in irofulven-induced FANCD2 monoubiquitination, GM00847 human fibroblasts expressing tetracycline-controlled, FLAG-tagged kinase-dead ATR (48) were used. Both doxycycline-induced and uninduced cells were treated with irofulven. As shown in Fig. 2B, the FLAG-tagged kinase-dead ATR was induced by doxycycline as determined by Western blot with anti-FLAG antibody. There was some constitutive FANCD2 monoubiquitination in untreated GM00847 cells. Upon irofulven treatment, there was an increase in FANCD2 monoubiquitination, the ratio of FANCD2-L to FANCD2-S increased from 0.9 to 2.3. When the kinase-dead ATR was induced by doxycycline in these cells, FANCD2 monoubiquitination was markedly blocked, the ratio of FANCD2-L to FANCD2-S was only slightly increased from 0.3 to 0.5 after irofulven treatment. Moreover, the overall FANCD2 monoubiquitination was also blocked. The ratio of FANCD2-L to FANCD2-S dropped from 0.9 to 0.3 in untreated cells; and from 2.3 to 0.5 in irofulven-treated cells (Fig. 2B).

To further confirm that ATR is important in mediating irofulven-induced FANCD2 monoubiquitination, an RNA interference approach was used to specifically knock-down the endogenous ATR expression in SKOV3 cells. The cells were infected with retrovirus containing short hairpin ATR or short hairpin GFP control construct, respectively. Western blot results indicated that the endogenous ATR level was knocked down by >60% as quantified by densitometry (Fig. 2C). Some constitutive FANCD2 monoubiquitination in untreated GM00847 cells. Upon irofulven treatment, there was an increase in FANCD2 monoubiquitination, the ratio of FANCD2-L to FANCD2-S increased from 0.9 to 2.3. When the kinase-dead ATR was induced by doxycycline in these cells, FANCD2 monoubiquitination was markedly blocked, the ratio of FANCD2-L to FANCD2-S was only slightly increased from 0.3 to 0.5 after irofulven treatment. Moreover, the overall FANCD2 monoubiquitination was also blocked. The ratio of FANCD2-L to FANCD2-S dropped from 0.9 to 0.3 in untreated cells; and from 2.3 to 0.5 in irofulven-treated cells (Fig. 2B).

To further confirm that ATR is important in mediating irofulven-induced FANCD2 monoubiquitination, an RNA interference approach was used to specifically knock-down the endogenous ATR expression in SKOV3 cells. The cells were infected with retrovirus containing short hairpin ATR or short hairpin GFP control construct, respectively. Western blot results indicated that the endogenous ATR level was knocked down by >60% as quantified by densitometry (Fig. 2C). Some constitutive FANCD2 monoubiquitination in untreated SKOV3 cells. In short hairpin GFP control-infected cells, the ratio of FANCD2-L to FANCD2-S increased from 0.7 to 1.7 after irofulven treatment. In short hairpin ATR-infected cells, FANCD2 monoubiquitination was greatly blocked, the ratio of FANCD2-L to FANCD2-S was 1.0 in untreated cells and 0.3 in irofulven-treated cells (Fig. 2C). Taken together, these results indicate that irofulven-induced FANCD2 monoubiquitination is mediated by ATR.

Figure 2. Irofulven-induced FANCD2 monoubiquitination was mediated by ATR. A, the vector and FLAG-tagged ATM-transfected AT fibroblasts (AT22JE-T-pEB57 and AT22JE-T-pEB57-YZ5) were treated with 5 μmol/L of irofulven for 1 h followed by 24 h of posttreatment incubation. Western blots were done with anti-FLAG and anti-FANCD2 antibodies. The ratio of FANCD2-L to FANCD2-S was determined by densitometry. B, GM00847 human fibroblasts expressing tetracycline-controlled, FLAG-tagged kinase-dead ATR were induced with doxycycline. The induced and uninduced cells were then treated with 5 μmol/L of irofulven for 1 h followed by 24 h of drug-free incubation. Western blots were done with anti-FLAG and anti-FANCD2 antibodies. The ratio of FANCD2-L to FANCD2-S was determined by densitometry. C, SKOV3 cells were infected with viral supernatants containing short hairpin GFP or short hairpin ATR, respectively. The short hairpin GFP was used as the nonspecific control. Forty-eight hours after infection, cells were treated with 2.3 μmol/L of irofulven for 1 h followed by 24 h of drug-free incubation. Western blots were done with anti-ATR and anti-FANCD2 antibodies. The ratio of FANCD2-L to FANCD2-S was determined by densitometry.
FANCD2 Is Important for Chromosome Integrity in Response to Irofulven

A characteristic feature of cells from patients with Fanconi anemia is the formation of chromosome aberrations (breaks and radials) following treatment with DNA cross-linking agents (1, 2). We have observed that irofulven induces FANCD2 monoubiquitination and nuclear foci formation. These findings, together with our previous observations that irofulven activates ATM and its target genes NBS1, SMC1, and CHK2 (44), suggest that irofulven induces DNA double-strand breaks. Therefore, we hypothesize that FANCD2 might play a pivotal role in maintaining chromosome integrity, thereby affecting chemosensitivity in response to irofulven.

To assess the role that FANCD2 might play in maintaining chromosome integrity upon irofulven treatment, mitotic spread experiments were done in the vector and wild-type FANCD2-transfected PD20F fibroblasts. Chromosome aberrations (breaks and radials) were observed after irofulven treatment (Fig. 3A), indicating that irofulven does induce DNA double-strand breaks. Chromosome breaks and radials were observed more frequently in vector-transfected than in FANCD2-transfected PD20F cells after irofulven treatment (Fig. 3B and C), suggesting that FANCD2 plays a critical role in repairing DNA damage and maintaining chromosome integrity in response to irofulven-induced DNA damage.

To further confirm the role that FANCD2 plays in maintaining chromosome integrity in response to irofulven, the RNA interference approach was again used. Three shFANCD2 constructs (sh-F1, sh-F2, and sh-F3) and the control vector (sh-V) were stably transfected into SKOV3 cells. The effectiveness of these three shFANCD2 constructs in knocking-down the endogenous FANCD2 was verified by Western blotting. As shown in Fig. 4A, sh-F1 is the most effective construct in knocking-down FANCD2, therefore, the sh-F1 and sh-V-transfected SKOV3 cells were used in subsequent studies.

When mitotic cells of the vector (sh-V) and shFANCD2 (sh-F1)-transfected SKOV3 cells were analyzed after irofulven treatment, chromosome breaks and radials were observed in vector-transfected cells (Fig. 4B and C). However, in sh-F1-transfected cells, more severe chromosome damage was observed. In these metaphase cells, chromosomes were damaged to the point where all of the chromosomes seemed to be broken or fragmented (Fig. 4B and C). These results further showed that FANCD2 is critical for maintaining chromosome integrity in response to irofulven-induced DNA damage.

FANCD2 Confers Chemoresistance to Irofulven

Based on the above observations that FANCD2 is activated and is important for chromosome integrity in response to irofulven-induced DNA damage, we hypothesize that FANCD2 might affect chemosensitivity to irofulven. To determine whether FANCD2 affects chemosensitivity to irofulven, the clonogenic survival assay was conducted. The vector, wild-type, or K561R mutant FANCD2-transfected PD20F fibroblasts were treated with different concentrations of irofulven for 1 h followed by drug-free incubations. When IC_{50} concentrations were compared, the results showed that FANCD2-transfected cells were 2-fold more resistant to irofulven than vector or K561R mutant FANCD2-transfected cells (Fig. 5A). This finding was further supported by the clonogenic
survival assay in vector and shFANCD2-transfected SKOV3 cells, where knocking-down the endogenous FANCD2 by RNA interference resulted in a >4-fold increase in chemosensitivity to irofulven when IC50 concentrations were compared (Fig. 5B). We also conducted a clonogenic assay with longer exposure times to verify that FANCD2-depleted cells were more sensitive. The results indicated that at 0.25 μmol/L, a concentration that caused no difference in chemosensitivity when used for treatment for only 1 h (Fig. 5B), the shFANCD2-transfected cells were ~4-fold or 24-fold more sensitive than the vector-transfected cells when treated for 6 or 24 h, respectively (Fig. 5C). Taken together, these results show that FANCD2 expression confers chemoresistance to irofulven, and FANCD2 monoubiquitination is the determining factor for FANCD2-mediated chemoresistance to irofulven.

Discussion
In this study, by using cell lines proficient or deficient for FANCD2, ATR, or ATM, we have shown that irofulven induces the monoubiquitination and foci formation of FANCD2. Irofulven-induced FANCD2 monoubiquitination is mediated by ATR. FANCD2 is critical for maintaining chromosome integrity and modulating chemosensitivity in response to irofulven.

There was some increase in the total amount of FANCD2 proteins observed in some cells following irofulven treatment (Fig. 1A). This could be the result of relatively overloading the protein extracts because it was not seen in other cells following irofulven treatment (Fig. 1A and C and Fig. 2B and C).

It has been reported that FANCD2 was phosphorylated by ATM or ATR in response to IR or psoralen/UV-A (52, 53). The phosphorylation of FANCD2 is required for radiation-induced S phase cell cycle checkpoint, but not chemoresistance to mitomycin C (52). In this study, a slight shifting of both FANCD2-L and FANCD2-S bands following irofulven treatment was also observed (Figs. 1 and 2), indicating that FANCD2 was also phosphorylated in response to irofulven-induced DNA damage. This suggests that FANCD2 might also play some role in irofulven-induced cell cycle arrest.

The Fanconi anemia-BRCA pathway of DNA damage response is frequently impaired in cancers due to genetic mutation or epigenetic repression (1, 2, 4, 9, 10, 19). The findings of this study suggest that FANCD2 activation might be used as a potential predictive marker for chemoresistance to irofulven therapy. Furthermore, the findings of this study also suggest that the genetic defects in other proteins of the Fanconi anemia-BRCA pathway may also be exploited for individualized therapy by irofulven. Further studies are warranted to determine whether these proteins will also influence the chemosensitivity to irofulven.

To date, the structure and nature of irofulven-induced DNA damage have not been characterized. Earlier studies suggested that the DNA damage caused by the illudin family of compounds might be repaired by the nucleotide excision repair pathway (41, 42). A recent study indicated that transcription-coupled nucleotide excision repair is the exclusive pathway in repairing illudin S and irofulven-elicited DNA lesions; base excision repair and nonhomologous end-joining of DNA double-strand break repair do not play a major role (43). However, in these studies, the homologous recombination pathway for double-strand break repair was not evaluated (41–43). Recent reports indicated that ATM and CHK2 are specifically activated by drug or radiation-induced DNA double-strand breaks (45, 46). Our previous studies showed that irofulven activates ATM and

Figure 4. Knocking-down FANCD2 by RNA interference results in increased chromosome aberrations. A, SKOV3 cells were stably transfected with the vector (sh-V) or shFANCD2 constructs (sh-F1, F2, and F3), respectively. The efficacy of shFANCD2 constructs in knocking-down the endogenous FANCD2 was determined by Western blot analysis. B and C, the vector (sh-V) and shFANCD2 (sh-F1)-transfected SKOV3 cells were treated with 2.3 μmol/L of irofulven for 1 h followed by 24 h of drug-free incubation. Mitotic spread staining was done. Arrows, chromosome breaks and radials (B). Columns, the mean percentage of mitotic cells with four or more chromosome breaks from triplicate counts of 50 cells; bars, SD (C).
its targets, NBS1, SMC1, CHK2, and p53 (44). These findings suggest that irofulven causes the generation of DNA double-strand breaks. In support of this, we show here that irofulven induces chromosome aberrations (breaks and radials) observed after irofulven treatment are also reminiscent of Fanconi anemia and BRCA2-deficient cells treated with the DNA cross-linking agent mitomycin C (1, 2, 21, 59). Therefore, it can be postulated that irofulven might cause stalled DNA replication leading to the generation of DNA double-strand breaks. In this study, we found that irofulven-induced FANCD2 monoubiquitination is mediated by ATR, and that FANCD2 monoubiquitination directly contributes to chemoresistance to irofulven. Therefore, it is conceivable that ATR is also activated by irofulven and plays an important role in modulating the chemosensitivity to irofulven. How ATR controls irofulven-induced FANCD2 monoubiquitination is currently unknown. It could be that ATR activates the essential upstream protein components or ubiquitin ligases that are critical for FANCD2 monoubiquitination. Further studies are needed to elucidate the precise mechanisms that lead to the generation of double-strand breaks, and how other DNA damage signaling proteins might control the chemosensitivity in response to irofulven.

In summary, we have observed that irofulven induces FANCD2 monoubiquitination/activation and foci formation. Irofulven-induced FANCD2 monoubiquitination is mediated by ATR. FANCD2 is crucial for maintaining chromosome integrity and modulating chemosensitivity in response to irofulven-induced DNA damage. These findings will enhance our understanding of the mechanisms of action involved with irofulven, and more specifically, the proteins and mechanisms that might affect chemosensitivity to irofulven. These findings will also provide insight for additional studies of targeted therapy by irofulven in cancers defective for proteins of the Fanconi anemia-BRCA pathway.

Acknowledgments

We thank Dr. Alan D’Andrea (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA) for generously providing the vector, wild-type, and K561R mutant FANCD2-transfected PD20F fibroblasts; Drs. S.L. Schreiber and S. Handeli for the GM00847 human fibroblast expressing kinase-dead ATR; and Dr. Y. Shiloh for the ATM-complemented AT fibroblast (AT22UE-T-pEBST7-YZ5). We also thank Dr. Linda Sargent for the use of microscopes, and Emily Van Laar and Shannon Wadman for critical reading of the manuscript.

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Molecular Cancer Therapeutics

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*Mol Cancer Ther* 2006;5:3153-3161.

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