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Abstract

Alteration of the Fragile Histidine Triad (*FHIT*) gene, encompassing the FRA3B fragile site at chromosome 3p14.2, especially reduction or deletion of its expression, is involved in many breast cancers. Little is known about the biological function of the Fhit in breast cancer progression. The major goal of this proposal is to test the hypothesis that the over-activated ATR pathway in irradiated $Fhit^{-/-}$ cells promote the homologous recombination repair (HRR) of DNA DSBs, resulting in more $Fhit^{-/-}$ cells surviving with deletion or translocations at fragile sites (malignant feature). In the passed year with this grant support, we found that the over-activated ATR pathway regulated checkpoint contributes the radioresistance of $Fhit^{-/-}$ cells and ATR is linked to HRR but not non-homologue end joining repair. These results will provide theory guidance for improving clinical treatment of breast cancers in which Fhit is deleted or reduced by combining traditional therapy with blocking the ATR pathway.

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INTRODUCTION:

Alteration of the Fragile Histidine Triad (*FHIT*) gene, encompassing the FRA3B fragile site at chromosome 3p14.2 (1), especially reduction or deletion of its expression, is involved in many solid tumors including breast cancer. It is reported that a complete loss or a significant reduction of Fhit expression was observed in 72% of breast carcinomas examined and a correlation between a decrease or an absence of Fhit protein expression and high proliferation and large tumor size of breast cancer was identified (2). Little is known about the biochemical and biological function of the Fhit in breast cancer progression. DNA double strand break (DSB) that may occur spontaneously and can be induced by DNA damage reagents such as ionizing radiation (IR) is one of the frequent reasons for promoting breast tumor genesis and development. The purpose of this proposal is to test our hypothesis that the over-activated ATR pathway in irradiated $Fhit^{-/-}$ cells promote the homologous recombination repair (HRR) of DNA DSBs, resulting in more $Fhit^{-/-}$ cells surviving with deletion or translocations at fragile sites (malignant feature).

BODY:

Two major pathways repairing DNA DSB exist in mammalian cells: non-homologous rejoining (NHEJ) and homologous recombination repair (HRR). By studying Fhit knockout cell lines obtained from Dr. Huebner's laboratory (3), we observed that $Fhit^{-/-}$ cells are more resistant to IR-induced killing than $Fhit^{+/+}$ cells. The radioresistant phenotype of $Fhit^{-/-}$ cells is associated with stronger S and G2 checkpoint response cells (Fig. 1) regulated by an over-activated ATR/CHK1 pathway (Fig. 2-4) (4). Also, we found that ATR and CHK1 that protecting cells from IR-induced killing is linked to HRR but not NHEJ (5, 6). In addition, we demonstrated that Fhit affecting cellular radiosensitivity is linked to HRR but independent of NHEJ (7). The results are briefly described in the following paragraphs.

MATERIALS AND METHODS

Cell lines, chemical treatment and irradiation. $Fhit^{+/+}$ and $Fhit^{-/-}$ epithelial cells from mouse kidney, generated as described earlier (3), were immortalized by tissue culture passaging. These cells were adapted to growth in DMEM supplemented with 10% iron-supplemented calf serum (Sigma-Aldrich Co. USA) at 37°C in an atmosphere of 5% CO_2 and 95% air. Caffeine (Sigma-Aldrich Co.), or UCN-01 (National Cancer Institute, USA) was added to the culture 30 min before the cells were exposed to X-rays (310 kV, 10 mA, 2-mm Al filter) and was kept in the culture until the cells were collected.

DNA synthesis. The S phase (S) checkpoint is detected by measuring DNA synthesis, which is similar to that described previously (8). Briefly, $1x10^5$cells from a growing culture were seeded in 60-mm tissue culture dishes with 3 ml of medium containing 10 nCi of [^{14}C]-thymidine and allowed to grow for more than one doubling time. This [^{14}C] pre-labeling provides an internal control for cell number by allowing normalization for total DNA content of samples.

Before irradiation, the cell cultures were changed with pre-warmed medium (washed off (^{14}C)-thymidine) containing either caffeine or UCN-01 for 30 min. Cells were exposed to X-rays (310 kV, 10 mA, 2-mm Al filter) at room temperature and returned to 37°C. The chemicals were kept in the culture until cells were harvested. (^{3}H)-thymidine at 0.5 μCi was added to the culture for 30 min at 3 h after IR and the cells were then collected. The rate of DNA synthesis for each sample was calculated as ^{3}H dpm/^{14}C dpm and is presented as a percentage of the control values obtained from sham-irradiated cells at the same time-point, as described previously (8).

Flow cytometry assay. The G2 checkpoint is detected by flow cytometry measurement. As described previously (9), Fhit cells were collected at required times and fixed in 70% ethanol. Cells were washed with PBS and stained with a solution containing 62 μg/ml RNase A, 40 μg/ml propidium iodide and 0.1% Triton X-100 in phosphate-buffered saline at room temperature for 1 h. The distribution of cells in the cell cycle was measured in a flow cytometer (Coulter Epics Elite, USA).

Kinase activity and western blot. Nuclear extracts were prepared by using the NE-PER^{TM} kit (PIERCE, USA) according to the manufacturer's instructions. The fractions of chromatin-bound extract were prepared as described previously (10). Briefly, cells were collected and washed in cold phosphate-buffered saline. Proteins were then extracted with cold 0.1% Triton X-100 in CSK buffer (10 mM PIPES pH 6.8, 100 mM NaCl, 300 mM sucrose, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfony fluoride) for 20 min at 4°C. The chromatin-bound fraction was then pelleted by low-speed centrifugation at 3,000 rpm for 5 min at 4°C. The supernatant was named fraction 1. These pellets were then re-extracted by incubating in the same CSK buffer and were collected by centrifugation at 3,000 rpm for 10 min at 4°C. This supernatant was named fraction 2. The final pellet fraction (containing chromatin-bound proteins) was solubilized in radioimmunopreciptation assay (RIPA) buffer (150 mM NaCl, 40 mM MOPS, pH 7.2, 1 mM EDTA, 1% NP40, 1% Sodium deoxycholate, 0.1% SDS) and was named fraction 3. For ATR kinase assay, 500 μg of fraction 3 was then mixed with 2 μg of ATR antibody (sc-1887, Santa Cruz Biotechnology, Inc. USA) in the presence of 20 μl of a 50% (v/v) protein G-Sepharose slurry (RepliGen, USA) in 500 μl of Buffer A (0.5% NP-40, 1 mM Na_3VO_4, 5 mM NaF, 0.2 mM PMSF in PBS buffer). For CHK1 kinase assay, 250 μg of nuclear extract was then mixed with 1 μg of CHK1 antibody (sc-7898, Santa Cruz Biotechnology, Inc.) in the presence of 10 μl of a 50% (v/v) protein A-Sepharose slurry in 250 μl of Buffer A. These mixtures were gently rotated overnight at 4°C. Immune complexes were washed twice with Buffer A, then twice with Buffer B (10 mM HEPES, pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT). The ATR kinase immunoprecipitate supplemented with 1 μg of PHAS-1 (Stratagene, USA) and the CHK1 kinase immunoprecipitate supplemented with 1 μg of purified GST-$CDC25C_{200-256}$ (10) were incubated at 30°C for 30 min in 20 μl Buffer B containing 10 μCi (γ-^{32}P) ATP. Samples were analyzed by 12% SDS-PAGE and the kinase activities determined by measuring the incorporation of ^{32}P into PHAS-1 protein (ATR kinase) or into $CDC25C_{200-256}$ (CHK1 kinase) with the PhosphoImager. Antibodies against ATR (sc-1887, Santa Cruz Biotechnology, Inc. USA), CHK1 (sc-

8404, Santa Cruz Biotechnology, Inc.) and CDC25A (sc-7389, Santa Cruz Biotechnology, Inc.) were used in the Western Blot.

Colony-forming assay. Cellular sensitivity to radiation was determined by the loss of colony-forming ability as described previously (9).

Induction and rejoining of DNA double strand breaks (DSBs). Analysis of induction and rejoining of DNA DSBs is performed by using the asymmetric field inversion gel electrophoresis (AFIGE) assay as described before (5). Briefly, cells in cold medium were irradiated and returned to the incubator at 37°C. At various times thereafter cells were collected and mixed with an equal volume of 1% agarose (InCert agarose, FMC). A similar protocol was also employed to determine induction of DNA DSBs except that in this case cells were embedded in agarose blocks prior to irradiation, and were placed in lysis buffer (10 mM Tris, pH 8.0, 50 mM NaCl, 0.5 M EDTA, 2% N-lauryl sarcosyl, 0.1 mg/ml proteinase E) immediately after irradiation. Blocks in lysis buffer were incubated first at 4°C for 45 min, and then at 50°C for 16-18 hrs. The blocks were washed in a buffer containing 10 mM Tris, pH 8.0 and 0.1 M EDTA, and treated at 37°C for 1 h with 0.1 mg/ml RNase A in the same buffer. AFIGE was carried out in 0.5% Seakem agarose (FMC) in 0.5X TBE (45mM Tris, pH 8.2, 45mM Boric Acid, 1mM EDTA) at 10°C for 40 h. During this time, cycles of 1.25 V/cm for 900 s in the direction of DNA migration alternated with 5 V/cm for 75 s in the reverse direction. The agarose gels were stained with ethidium bromide (5 μg/ml) for 6 h at room temperature and washed with H_2O for 1 h. DNA DSBs were quantitated by calculating the FAR (fraction of activity released from the well into the lane) in irradiated and non-irradiated samples by means of a fluorescence image measured with a PhosphoImager (Typhoon 8600, Molecular Dynamics).

HRR Assay. The HRR assay was performed using the pDR-GFP system (obtained from Dr. Jasin's laboratory) (11). Fhit wild type cells were transfected with pDR-GFP plasmid containing a mutated GFP gene with an 18 bp I-SceI site. The stably transfected cell lines were selected by growing in medium containing 5 μg/ml of puromycin. Puromycin-resistant colonies were screened by Southern blots for intact DR-GFP reporter genomes. The positive cell lines were designated F-DRGFP. To evaluate HRR of DNA DSBs, F-DRGFP cells were either transfected with pGFP (containing full-length GFP cDNA) or transfected with pCMV3xnlsI-SceI plasmid (containing full-length I-SceI expression sequences, obtained from Dr. Nickoloff's laboratory (12)). Transient expression of I-SceI endonuclease generates a DSB at the integrated GFP gene sequences and stimulates HRR. GFP signal was assayed at two days post transfection by flow cytometry (Beckman Coulter, XL/MCL). Results were collected as dot plots of PMT1 (525 BP) to facilitate distinction between GFP positive and GFP negative cells. The frequency of recombination events was calculated from the frequency of GFP signal in F-DRGFP cells transfected with I-SceI by subtracting the frequency of GFP signal in F-DRGFP cells without transfection and dividing by the frequency of GFP signal in F-DRGFP cells transfected with pGFP.

Transfection of *Atr*, *Chk1* or *Fhit* siRNA. The *Atr* siRNA was designed to specifically target the sequences of the conserved region between rat and mouse *Atr* mRNA (5'-AAGACAGATTCTCTGCCAGTT-3'). The *Chk1* siRNA was designed to specifically target the sequences of the conserved region among human, rat and mouse *Chk1* mRNA (5'-AAGTTCAACTTGCTGTGAATA-3'). *Fhit* siRNA was designed to specifically target the sequences of the mouse *Fhit* mRNA (5'-AAGCAUUUCCAGGGGACCUCC-3'). The siRNAs were synthesized by Dharmacon, Inc. Scrambled duplex RNAs (Dharmacon, Inc. USA) were used in the control transfection. The RNAs were delivered to the cells by OLIGOFECTAMINE^{TM} (Invitrogen Corp. USA), according to the manufacturer's instructions. The cells were analyzed at 36 h posttransfection.

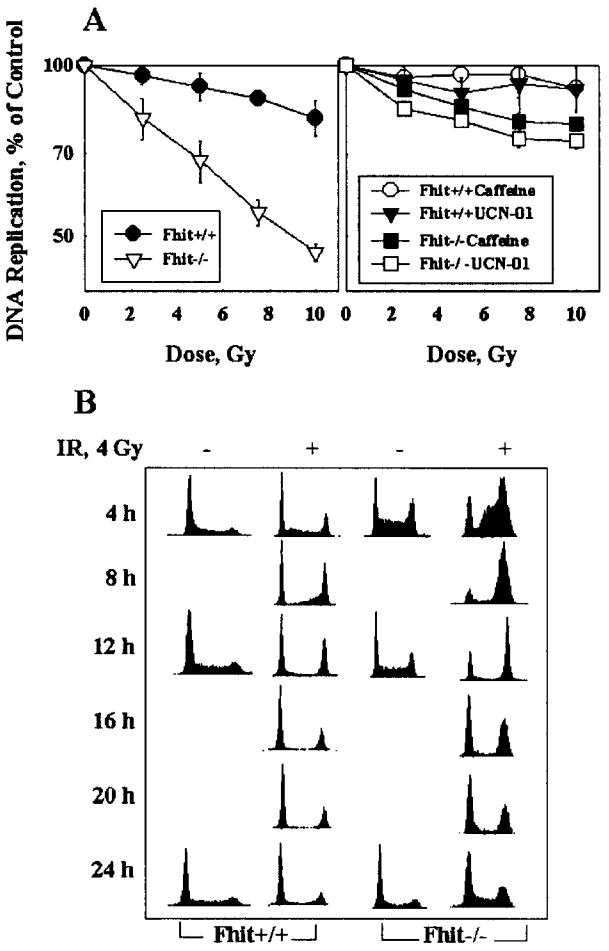


Fig. 1. **Stronger S and G2 checkpoint responses are shown in irradiated Fhit$^{-/-}$ cells. A:** DNA replication of Fhit cells following IR. ^{14}C-Tdr pre-labeled cells received pre-warmed medium with or without treatment of caffeine (4 mM) or UCN-01 (100 nM) for 30 min before IR. At 3 h after exposure to different doses of IR, 0.5 μM ^{3}H-Tdr was added to the cell cultures. Thirty min later the cells were collected and loaded on GF-A filters set in a Millipore Vacuum chamber. The following procedures are as described previously (8). The rate of DNA synthesis for each sample was calculated as ^{3}H dpm/^{14}C dpm and is presented as a percentage of the control values obtained from sham-irradiated cells at the same time-point. The data are presented as mean values and standard deviations from three independent experiments. **B:** G2 arrest in Fhit cells following IR. The Fhit cells were collected at indicated time-points after exposure to 4 Gy and fixed in 70% ethanol. Cells were washed with PBS and stained with the solution (62 μg/ml RNase A, 40 μg/ml propidium iodide and 0.1% Triton X-100 in phosphate-buffered saline) at room temperature for 1 h. The distribution of cells in the cell cycle was measured in a flow cytometer (Coulter Epics Elite).

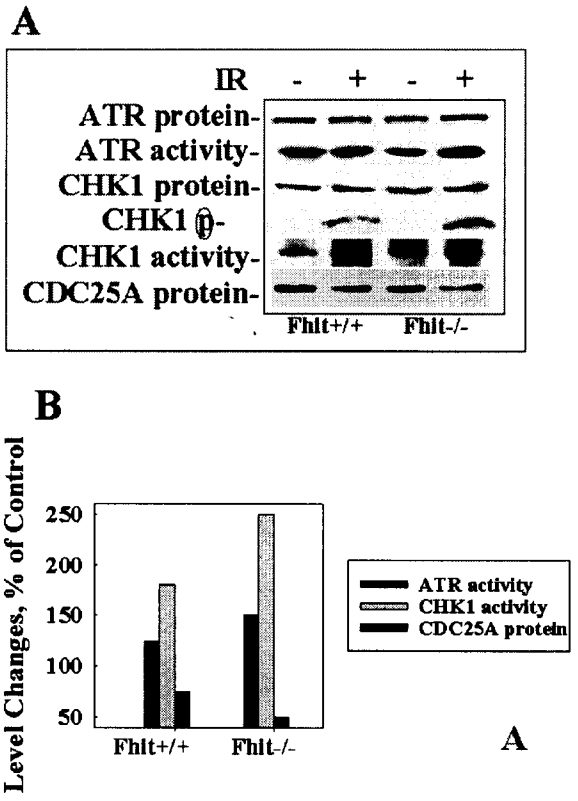


Fig. 2. **An over-activation of the ATR/CHK1 pathway is shown in irradiated Fhit$^{-/-}$ cells. A:** The Fhit cells were collected 6 h after IR (10 Gy). Protein levels were measured by using nuclear extracts from non-or irradiated Fhit cells. The kinase activities were measured in ATR or CHK1-immunoprecipitated nuclear extracts according to the protocols described in "Materials and Methods". **B:** Activity levels were obtained by quantifying ATR and CHK1 activities, as well as CDC25A levels shown in (**A**), using the PhosphoImager software. Similar results were obtained from two independent experiments.

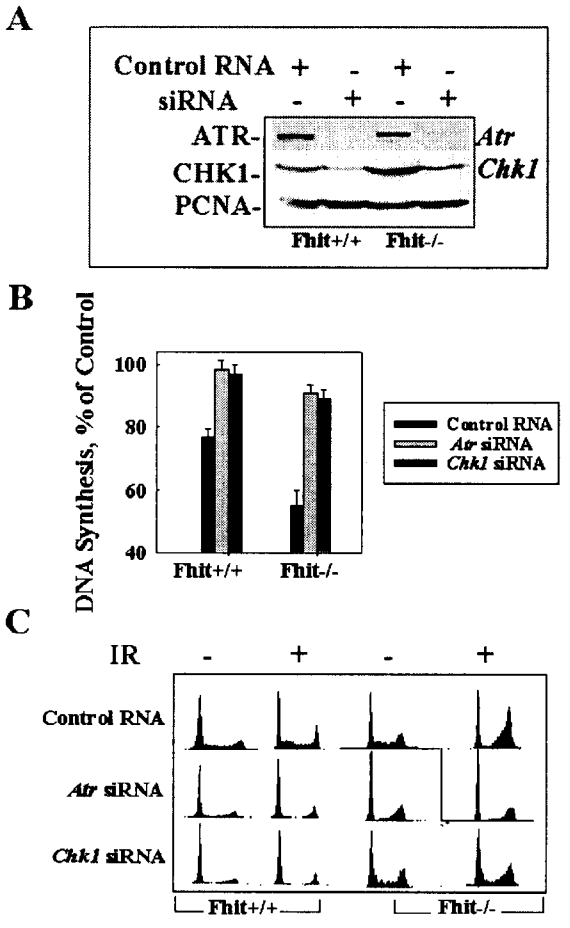


Fig. 3. ***Atr* or *Chk1* siRNA abolishes the stronger S and G2 checkpoint responses in irradiated Fhit$^{-/-}$ cells. A**: The levels of ATR and CHK1 expression were measured with the extracts from either siRNA of *Atr* and *Chk1* antisense or control RNA treated Fhit cells. PCNA was used as the internal control. **B**: DNA replication measured in *Atr* or *Chk1* siRNA treated cells following IR. The treatments of *Atr* or *Chk1* siRNA are as described in "Materials and methods". The Fhit cells were collected at 3 h after 4 Gy of exposure. The ratios of DNA replication rates are presented as mean values and standard deviations from three independent experiments. **C**: G2 arrests measured in *Atr* or *Chk1* siRNA treated cells following IR. As described in "Materials and methods", Fhit cells were treated with siRNA of *Atr* or *Chk1* for 36 h and then irradiated (2 Gy). At 6 h after IR, cells were collected for preparation and measurement of flow cytometric profiles of cell cycle distribution as described in Figure 1.

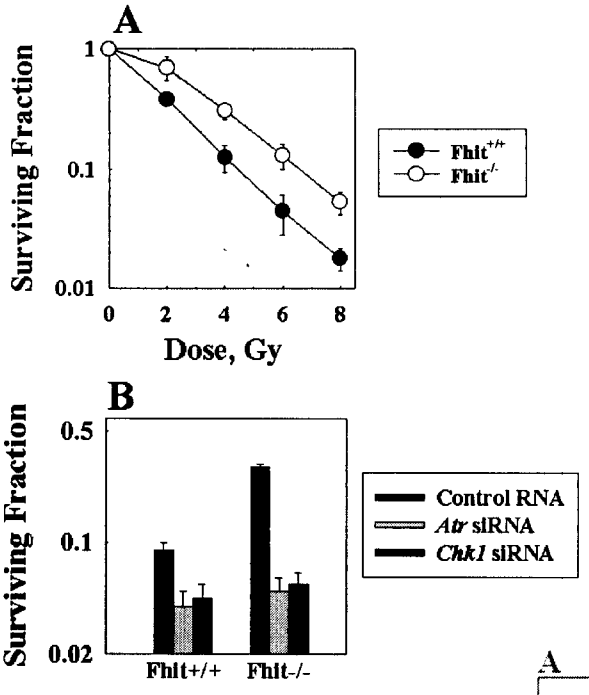


Fig. 4. **The radioresistance of Fhit$^{-/-}$ cells is diminished by *Atr* or *Chk1* siRNA treatment. A:** Cellular sensitivity to radiation was determined by the loss of colony-forming ability as described previously (9). Data shown are the average from three independent experiments. **B:** *Atr* or *Chk1* siRNA sensitized Fhit cells to IR-induced killing. At 36 h after siRNAs treatment cells were exposed to 4 Gy, then trypsinized and planted in medium without oligonucleotide. Data shown are the average from three independent experiments.

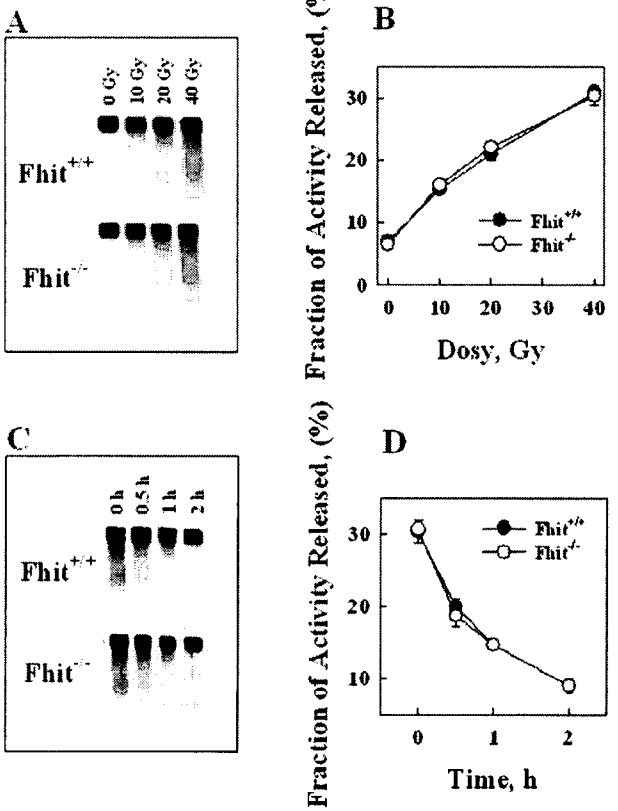


Fig 5. **Fhit$^{+/+}$ and Fhit$^{-/-}$ cells show similar DSB induction levels and similar DSB rejoining rates. A** and **B** show the induction of DNA DSBs. **C** and **D** show the kinetic rejoining of DNA DSBs after exposure to 40 Gy X-ray as described above. **B** and **D** are the quantification of the gel results shown in **A** and **C**. DNA DSBs were quantified by calculating the FAR (fraction of activity released from the well into the lane) in irradiated and non-irradiated samples by means of a fluorescence image measured with a PhosphoImager (Typhoon 8600, Molecular Dynamics). Data shown are the average and standard error from three independent experiments.

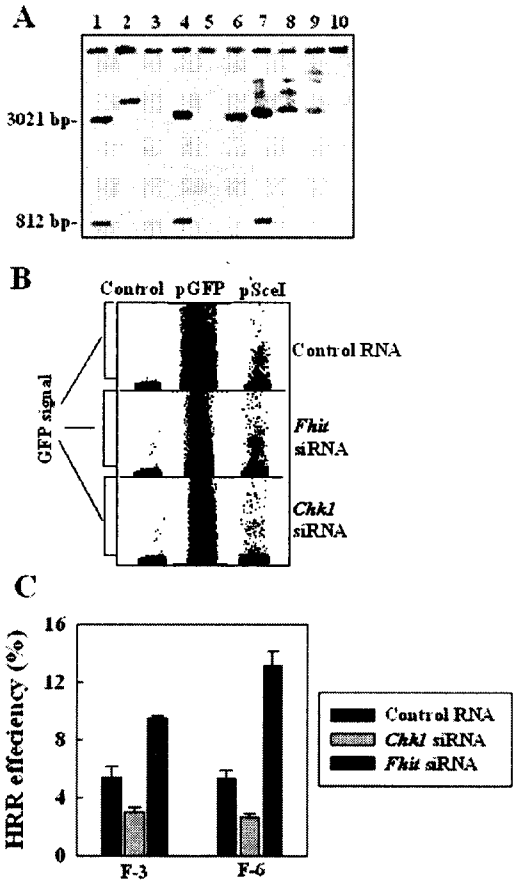
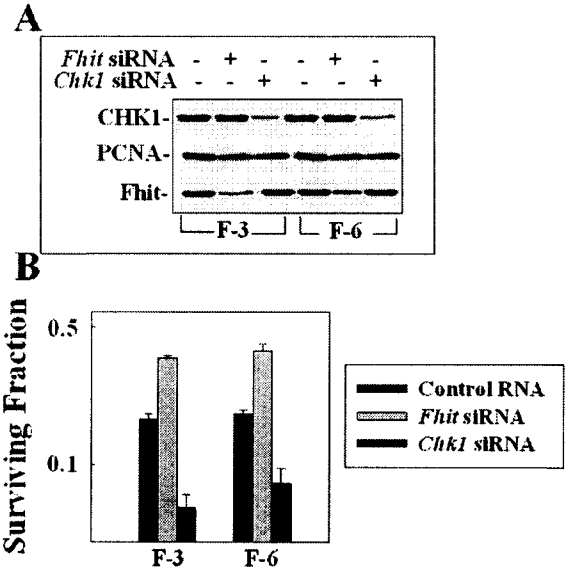


Fig 6. ***Fhit* knock down promotes HRR and *Chk1* knock down inhibits HRR. A.** Southern blot analysis of clones derived from transfection of the Fhit$^{+/+}$ cells with DR-GFP substrate. Genomic DNA from puromycin-resistant clones was digested with *Sal I* and *Hind III*, 3021 and 812bp GFP coding fragments were detected with a radiolabeled GFP probe as described (5). Lane 1 is the positive control obtained from Draa-40 (hamster cells) transfected with pDR-GFP (11). Lane 2-lane 10 are clones 1, 2, 3, 4, 5, 6, 7, 8 and 9. Clones 3 and 6 contain the GFP fragments and are named as F-3 (F-DRGFP-clone3) and F-6 (F-DRGFP-clone6) respectively. **B.** Representative flow cytometric analyses of GFP signal in F-6 cells with either *Fhit* or *Chk1* siRNA treatment following DSBs. The cells were either transfected with pGFP (alone or co-transfected with siRNA) or pDR-GFP (alone or co-transfected with siRNA) with Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Twenty-four h later, the cells were transfected with either Fhit siRNA or Chk1 siRNA by using the oligofectimine (Invitrogen) following the manufacturer's instructions. The cells were collected at 36 h following the siRNA transfection. **C.** HRR efficiency was calculated by the frequency of GFP signal in I-Sce I transfected F-DRGFP cells subtracting the background from control RNA-treated F-DRGFP cells and divided by the frequency of GFP signal from pGFP transfected F-DRGFP cells. Data shown are the average standard error from three independent experiments.

Fig 7. ***Fhit* knock down increases cells radioresistance and *Chk1* knocking down decreases cells radioresistance. A.** The levels of Fhit and CHK1 expression were measured with whole cell lysates from either *Fhit* siRNA or *Chk1* siRNA treated F-DRGFP cells. Thirty-six h after transfection, the cells were collected for measuring protein levels by Western blot. PCNA was used as the internal loading control. **B.** Cellular sensitivity to radiation was determined by the loss of colony-forming ability as described in the *Materials and Methods*. Thirty-six h after siRNA transfection, the cells were exposed to X-rays (4 Gy) and then collected for clonogenic assay. Data shown are the average from three independent experiments.



KEY RESEARCH ACCOMPLISHMENTS:

We reported that Fhit$^{-/-}$ cells are more resistant to IR-induced killing than Fhit$^{+/+}$ cells. The radioresistant phenotype of Fhit$^{-/-}$ cells is associated with stronger S and G2 checkpoint responses cells regulated by the over-activated ATR/CHK1 pathway (4). Also, we reported that ATR and CHK1 protect cell from IR-induced killing is linked to HRR but not NHEJ (5, 6). In addition, we demonstrated that Fhit affects cellular radiosensitivity is linked to HRR but independent of NHEJ (7).

REPORTABLE OUTCOMES:

Manuscripts:

1. Hu, B., Han, S-Y., Wang, X., Ottey, M., Potoczek, M.B., Dicker, A., Huebner, K., and **Wang, Y.** Involvement of the *Fhit* gene in the ionizing radiation-activated ATR/CHK1 pathway. J. Cell Physiol. 202: 518-523, 2005.
2. Hu, B., Wang, H., Wang, X., Lu, H-R, Huang, C., Powell, S.N., Huebner, K. and **Wang, Y.** Fhit and CHK1 have opposing effects on homologous recombination repair. Cancer Res. Accepted, 2005.

Abstract:

Hu, B., Han, S-Y., Wang, X., Ottey, M., Potoczek, M.B., Dicker, A., Huebner, K., and **Wang, Y.** Involvement of the *Fhit* gene in the ionizing radiation-activated ATR/CHK1 pathway. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Proceedings: P 212, 2005

Presentation

Involvement of the *Fhit* gene in the ionizing radiation-activated ATR/CHK1 pathway. Invited Speaker in Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Philadelphia, June 8-11, 2005:

Developed siRNAs:

1. ATR (4)
2. Chk1 (4)
3. Fhit (7)

Funding applied for based on work supported by this award:

One grant entitled "Characterization of the role of Fhit in maintenance of genomic integrity following low dose radiation, *in vivo* and *in vitro*" is funded by the Department of Energy. This project is to address research to elucidate the mechanism by which Fhit maintains genomic integrity by affecting checkpoint and DNA repair in *vitro* and *in vivo*, following low dose ionizing radiation (IR).

CONCLUSIONS:

These findings provide an explanation for the relationship between Fhit expression and breast cancer progression. The importance of this research is that it will provide theory guidance for improving clinical treatment of breast cancers in which Fhit is deleted or reduced by combining traditional therapy with blocking the ATR/CHK1 pathway.

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