# Chromosomal instability syndromes are sensitive to poly ADP-ribose polymerase inhibitors

Terry J. Gaymes, Sydney Shall, Farzin Farzaneh, and Ghulam J. Mufti

Department of Haematological Medicine, Leukaemia Sciences Laboratories, The Rayne Institute, Kings College London, Denmark Hill Campus, London, UK

## ABSTRACT

Poly ADP-ribose polymerase inhibitors have been shown to target cells with homologous recombination DNA repair defects. We report that poly ADP-ribose polymerase inhibitors induces apoptosis in cells deficient in other key DNA repair components. Chromosomal instability disorders, Fanconi Anemia and Bloom's syndrome have dysfunctional DNA repair and an increased likelihood of leukemic transformation. PI addition to Fanconi Anemia and Bloom's syndrome cells resulted in significant apoptosis. Furthermore, poly ADP-ribose polymerase inhibitors induced apoptosis in DNA repair signaling defective *ATM*$^{-/-}$ and *NBS*$^{-/-}$ fibroblasts. Immunocytochemistry showed homologous recombination was abrogated in *NBS*$^{-/-}$ and *ATM*$^{-/-}$ fibroblasts, compromised in Fanconi anemia and normal in Bloom's syndrome cells in response to poly ADP-ribose polymerase inhibitors. Strikingly, poly ADP-ribose polymerase inhibitors increases non-homologous end joining repair activity, whilst non-homologous end joining deficient cells are extremely sensitive to poly ADP-ribose polymerase inhibitors. These data suggest poly ADP-ribose polymerase inhibitors target cells with DNA repair and signaling defects rather than solely defects in homologous recombination improving the potential of poly ADP-ribose polymerase inhibitors therapy in a wider range of cancers.

Key words: Poly ADP-ribose polymerase, leukemia, DNA repair.

*Citation: Gaymes TJ, Shall S, Farzaneh F, and Mufti GJ. Chromosomal instability syndromes are sensitive to poly ADP ribose polymerase inhibitors. Haematologica 2008; 93:1886-1889. doi: 10.3324/haematol.13201*





## Introduction

A major feature of hematologic malignancies such as myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) is the presence of marked chromosomal instability. Recent data has highlighted the role of the double strand break (DSB) DNA damage signaling network as a cause of chromosomal instability in hematologic disorders.[1] In this regard, chromosome instability disorders such as Bloom's syndrome (BS) and Fanconi anemia (FA) have an increased propensity to transform to MDS and AML.[1,2] The aberrant genes in these syndromes are components of the homologous recombination (HR) pathway of DSB DNA repair. Additionally, mutations in other DSB DNA repair signaling components such as the ataxia telangiectasia mutated gene (*ATM*), the Nijmegen syndrome gene (*NBS1*), and DNA ligase IV (DNL IV)[3-5] have been identified in a percentage of leukemias.

Poly ADP-ribose polymerase (PARP) is a single strand break (SSB) sensing protein that catalyses the addition of ADP ribose to surrounding histones and other nuclear proteins.[6] Inhibitors of PARP have been shown to selectively target cells with a dysfunctional homologous recombination (HR) pathway of DSB DNA repair.[7] As a result of PARP inhibition, accumulation of single strand DNA breaks (SSB) leads to the replication fork collapse and conversion of SSB to double strand breaks DNA (DSB). The inability of repair defective cells such as *BRCA2*$^{-/-}$, *BRCA1*$^{-/-}$ and *ATM*$^{-/-}$ mutants to repair the DSB DNA breaks would lead to cell death.[8,9] In this report we show that PARP inhibitors induce cell death in chromosomal instability syndromes that have little or no defect in HR.

## Design and Methods

PARP Inhibitor PJ34 ($IC^{50}$: 30 nM) was purchased from Calbiochem, Nottingham, UK. The PARP inhibitor, KU-0058948 was donated by Kudos Pharmaceuticals, Cambridge, UK. Mouse VC-8 (*BRCA2*$^{-/-}$) and its isogenic control, V79-2 fibroblastic cell lines were provided courtesy of Margaret Zdzienicka, Leiden. Mouse lines were cultured in DMEM supplemented with 10% fetal bovine serum, 4 μM glutamine, 1% penicillin/streptomycin. Retrovirally immortalized PD220Di (human

*Funding: this work is funded by the National Health Service (NHS).*
*Manuscript received April 8, 2008. Revised version arrived on July 10, 2008. Manuscript accepted on July 15, 2008.*
*Correspondence: Ghulam J. Mufti, Department of Haematological Medicine, Kings College London, The Rayne Institute, 123 Coldharbour Lane, London SE5 9NU, UK. E-mail: ghulam.mufti@kcl.ac.uk*
*The online version of this article contains a supplementary appendix.*

*FANCD2*$^{-/-}$) and its isogenic corrected control, PD220Di +D2 fibroblastic cell lines, GM05849; human SV40 immortalized *ATM*$^{-/-}$ fibroblasts, GM16088; human SV40 immortalized *DNL IV*$^{-/-}$ fibroblasts, GM06914; human SV40 immortalized *FANCA*$^{-/-}$ fibroblasts, GM15989; SV40 immortalized NBS$^{-/-}$ fibroblasts and GM00637(GM); normal human SV40 immortalized fibroblasts were purchased from the Coriell Institute for Medical Research, Camden, NJ, USA. All human SV40 immortalized fibroblasts were derived from individuals aged 12-20. Cells were cultured in EMEM supplemented with 10% fetal bovine serum, 4 mM glutamine, 1% penicillin/streptomycin. SV40 immortalized PSNV4 (BS cells, *BLM*$^{-/-}$) and PSNF5 (*BLM* corrected) cell lines respectively, were cultured as previously described.[10]

### Immunofluorescence studies

Cells were immunostained and visualized as previously described.[11]

### Cell cycle analysis

Cells were prepared and analyzed as previously described.[11] Samples were analyzed using the FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). An apoptotic index was derived by calculating the percentage sub-$G_1$ population events as a fraction of the total sub-$G_1$ + $G_1$ population events.

Preparation of nuclear extracts was performed as described.[12]

End-ligation and misrepair assays were conducted as previously described.[12] Densitometry readings were taken for all ligated plasmid products. End-ligation efficiency was defined as the sum of the densitometry readings for all ligated products divided by the sum of the densitometry readings for all ligated products plus unligated plasmid.

For the misrepair assay, 1 μg of *Eco*R1 linearized pUC18 was incubated with saturating levels of nuclear extract (10 μg) to promote plasmid recirculization. Plasmid and nuclear extract was incubated for 24 hrs. at 18°C before being used to transfect *Escherichia coli* strain DH5α. Primers around the *Eco*R1 site were designed to give a PCR product of 628bp corresponding to nucleotides 150-777bp. Colony PCR was performed on blue and white colonies to determine the size of the deletion.

Soft agar clonogenic cell survival assays were conducted as described.[8] Log relative survival was calculated as clonogenic survival relative to the clonogenic survival in the cell line that produced the greatest number of colonies at that concentration of inhibitor. The maximum number of colonies at any given concentration for this cell line would be regarded as 100% and survival of clones in response to inhibitor for other cell lines would be made relative to this value.

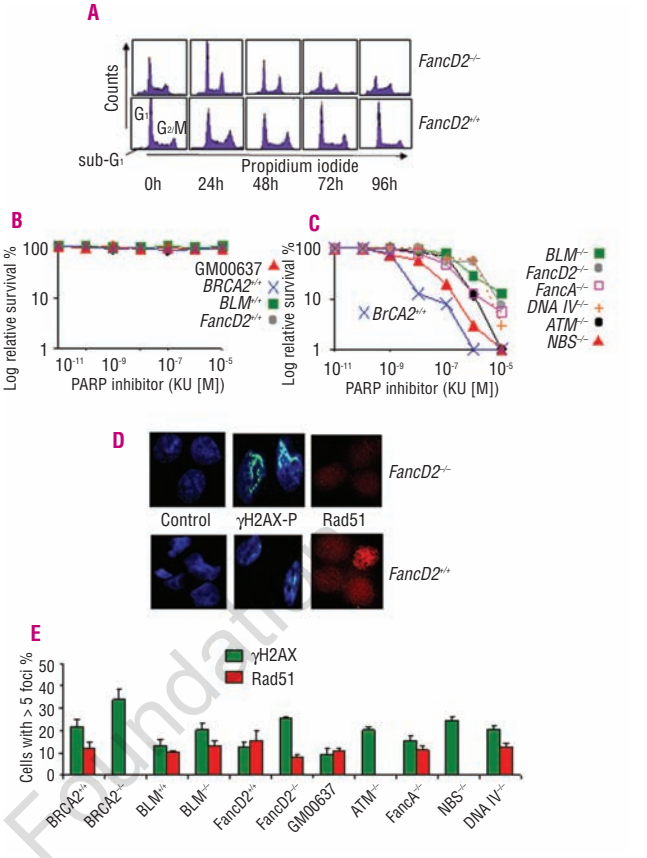


**Figure 1. The effect of PARP inhibitors on chromosomal instability cell lines that are defective in DNA repair. (A) 1 μM PJ34 was added to PD220Di (*FANCD2*$^{-/-}$) or PD220Di+D2 (*FANCD2*$^{+/+}$ corrected) cells for 96 hrs. and analyzed by flow cytometry, n=3. (B,C) The effect of PARP inhibitors on cell survival was assessed by a soft agar clonogenic assay, n=3. Cells were exposed to PARP inhibitors continuously for 12-14 days. KU-0058948 was added at variable concentrations to B, control fibroblast cell lines, GM00637 (triangles), PD220Di + D2 (*FANCD2* corrected) (gray circles), PSNF5 (*BLM* corrected) (green squares) and V79-2 (control mouse fibroblasts, isogenic for VC-8) (blue crosses) and in C, chromosomal instability fibroblast cell lines *ATM*$^{-/-}$ (black circles), PSNV4 (*BLM*$^{-/-}$) (green squares), FANCA-/- (open boxes, *DNL IV*$^{-/-}$ (crosses, dashed line), *FANCD2*$^{-/-}$ (gray circles), *NBS*$^{-/-}$ (triangles), VC-8 (mouse *BRCA2*$^{-/-}$ fibroblasts)(blue crosses). (D, E) Immunostaining of nuclei from chromosomal instability syndrome and control cells treated with 1 μM KU-0058948 for 24 hrs. Cells were probed for phosphorylated gH2AX foci (green) and rad51 foci (red). E, Frequency of cells displaying phosphorylated γH2AX foci (%) (green bars) or rad51 foci(%) (red bars), by immunofluorescence following KU-0058948 addition. More than 300 nuclei were counted per experiment, n=3. Error bars: SEM.**

## Results and Discussion

We investigated the possibility that chromosomal instability syndrome cells might show exaggerated hyper-sensitivity to PARP inhibitors (PI) by adding PJ34 or KU-0058948 to a panel of exponentially growing chromosomal instability cell lines. *FANCD2*$^{-/-}$(PD220Di) cells were cultured with 1 μM PJ34 and Figure 1A shows that these cells displayed a reduced $G_1$ population, an increased $G_2$/M population (indicative of stalled replication) and an increased sub-$G_1$ population (apoptotic population) by 96 hrs. (*Online Supplementary Appendix Table 1*). In contrast, the corrected cell, *FANCD2*$^{+/+}$ (PD220Di + D2) shows only reduced $G_1$, and increased $G_2$/M phase populations that return to a normal cell cycle profile by 96 hrs. consistent with the appropriate reactivation of replication. All chromosomal instability syndromes studied demonstrated abnormal cell cycle profiles culminating in an increased sub-$G_1$ population in contrast to isogenic controls and

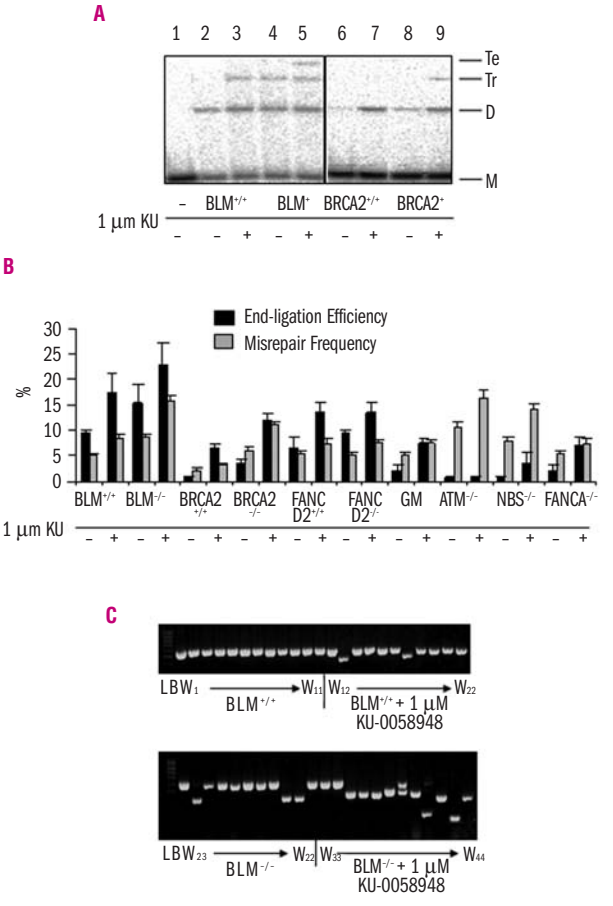


**Figure 2. PARP inhibitors increase NHEJ efficiency and inaccurate repair. (A) Plasmid ligation assay. Lane 1, plasmid only, Lane 2; *BLM*$^{+/+}$, Lane 3; *BLM*$^{+/+}$ + 1 μM KU-0058948. Lane 4; BLM$^{-/-}$, Lane 5; *BLM*$^{-/-}$ + 1 μM KU-0058948, Lane 6; *BRCA2*$^{+/+}$, Lane 7; *BRCA2*$^{+/-}$ + 1 μM KU-0058948, Lane 8; *BRCA2*$^{-/-}$, Lane 9; *BRCA2*$^{-/-}$ + 1 μM KU-0058948. End-ligation efficiency was defined as ligated products as a fraction of unligated plasmid + all products. M-monomer, D-dimer, Tr-Trimers, Te-Tetramers. (B) End-ligation efficiency (black bars) and misrepair frequencies (gray bars) (n=3). Error bars = SEM. (C) PCR of white colonies (W), untreated *BLM*$^{+/+}$ (W1-11), *BLM*$^{+/+}$ + 1 μM KU-0058948 (W12-22), Untreated *BLM*$^{-/-}$ (W23-32), *BLM*$^{-/-}$ + 1 μM KU-0058948 (W33-44). L- ladder, B-blue colony (628bp).**

control fibroblasts (*Online Supplementary Figure S1 and Table S1*). The clonogenic survival assay (Figure 1B,C) revealed that FA cells (*FANCD2*$^{-/-}$, *FANCA*$^{-/-}$), *BLM*$^{-/-}$, *BRCA2*$^{-/-}$, *ATM*$^{-/-}$, *NBS1*$^{-/-}$ and *DNL IV*$^{-/-}$ cells showed great sensitivity to PI compared to controls (Figure 1 B,C). *BRCA2*$^{-/-}$ cells were 10-fold more sensitive than *ATM*$^{-/-}$ and *NBS1*$^{-/-}$ cells that were in turn significantly more sensitive to PI than *FANCD2*$^{-/-}$, *FANCA*$^{-/-}$ or *DNL IV*$^{-/-}$ cells ($p$<0.01, n=3). Fanconi Anemia (FA) is a rare chromosomal instability disorder characterized by a hypersensitivity to DNA inter-strand cross-linking by alkylating agents and an increased likelihood of leukemic transformation.[1] FA gene *FANCA* has been previously identified as having reduced expression[13] or point mutations[14] in a number of adult AML patients. Mutations in *ATM, NBS1* and *DNL IV* have also been previously identified in leukemia.[3-5] The observation that the lack of *FA, ATM, NBS1* and *DNL IV* resulted in sensitivity to PARP inhibition further suggests that PARP inhibition could be of therapeutic benefit in a variety of hematologic malignancies with dysfunctions in DNA damage response genes. Immunocytochemical analysis was used to determine if PARP inhibitor sensitivity was attributable to defective homologous recombination (HR) DNA repair (Figure 1D,E). HR factor, Rad51 locates to areas of DNA damage forming nuclear foci, whilst phosphorylation of histone variant H2AX is a marker of DSB DNA damage. In response to adding 1 μM KU-0058948 for 24 hrs., *BRCA2*$^{-/-}$, *ATM*$^{-/-}$, *NBS1*$^{-/-}$ cells showed no rad51 foci formation, *FANCD2*$^{-/-}$, *FANCA*$^{-/-}$ showed reduced, but still prominent rad51 foci, whilst *BLM*$^{-/-}$ and *DNL IV*$^{-/-}$ cells demonstrated equivalent rad51 foci frequency compared to normal controls ($p$<0.05, n=3). The decreased but not absent rad51 foci formation in FA cells suggests that even though FA proteins are intrinsically involved in inter-strand crosslink repair and restart of DNA replication, FA has a *mild* HR defect as reported by other groups.[15,16] In fact, FA sensitivity to PI could be attributable to cell cycle checkpoint anomalies.[17] *BLM*$^{-/-}$ cells were also highly sensitive to PI, but had normal rad51 foci formation in response to PI challenge. It is suggested that the helicase action of BLM protein is required to suppress HR following a stalled replication fork by dissolving d-loops[18] and holliday intermediates,[19] and promoting replication fork reversal and eventual restart of replication. Thus BLM is an anti-HR factor and loss of BLM results in increased HR and elevated sister chromatid exchange.[20] As HR is functional in FA and BS cells, this suggests that absence of HR is not an absolute requirement for PI sensitivity. Rather, it is the absence of efficient and accurate repair of DSB that is required. We investigated the possibility that PARP inhibitors might influence the NHEJ DNA repair pathway.

Nuclear extracts were prepared from control and chromosomally unstable fibroblast lines that had been incubated with 1 μM KU-0058948 for 24 hrs. Figure 2 A,B and *Online Supplementary Figure S1* show that end-joining efficiency is significantly enhanced in PI treated cells (n=3, $p$<0.001) compared with untreated cells. However, in *ATM*$^{-/-}$ and *NBS*$^{-/-}$ cells that showed a significant reduction in end joining activity compared to GM control cells there was no significant change in end-ligation efficiency upon PI challenge. We next sought to determine if PI administration is associated with an increased frequency of errors of end-joining repair, since the NHEJ pathway is characterized as an error-prone repair pathway. For the most part the NHEJ pathway repairs DSB correctly, but it can also introduce errors in the form of small DNA deletions of less than 30bp during repair.[12]

All cells were treated with 1 μM KU-0058948 for 24 hrs., nuclear extracts were prepared from treated cells and used in a *lacZ* plasmid reactivation assay; Figure 2B,C show that PI treated control cells demonstrated increased misrepair frequency compared with untreated cells (GM, 5.2 *vs.* 7.6%; *BRCA2*$^{+/+}$ 2.2 *vs.* 3.3%; *BLM*$^{+/+}$, 5.2 *vs.* 8.3%) (n=3, $p$<0.01). Significantly, misrepair frequency is dramatically increased in chromosomally unstable cell lines treated with PI (*BLM*$^{-/-}$, 8.7 *vs.* 15.9%; *BRCA2*$^{-/-}$, 6.1 *vs.* 11.9%; *ATM*$^{-/-}$, 10.1 *vs.* 16%; *NBS*$^{-/-}$, 8 *vs.* 14.2%). More than 50 white colonies per experiment were randomly chosen from the test plates and were analyzed for plas-

mid deletions using PCR. PI treated *BLM*$^{-/-}$ cells elicited a much higher percentage of large plasmid deletions (35 to 400 bp) compared to untreated *BLM*$^{-/-}$ cells (30% *vs.* 85%) (Figure 2C). The NHEJ pathway works complementary to HR, although many have considered it the major pathway of DSB DNA damage repair.[10,21] However, HR remains the pathway of choice at S-phase to assist in the restoration of stalled replication forks. This is exemplified by the extreme sensitivity of HR deficient cells such as *BRCA2*$^{-/-}$ to PI challenge. Interestingly, we show that PI is able to increase NHEJ activity, suggesting that NHEJ is activated as a back-up/salvage mechanism in response to PI induced stalled replication. Addition of PI increases the misrepair (error) frequency of the error-prone NHEJ pathway, and this misrepair is further enhanced in HR defective cells. As we and others have demonstrated erroneous NHEJ repair of DSB in *BS* and *FA* cells, it is tempting to suggest that PI sensitivity in chromosomal instability syndromes is partially the result of increased genomic instability generated by an over-stimulated NHEJ pathway compensating for the loss of HR competency.[10,21] Thus, as cells deficient in NHEJ (*DNL IV*$^{-/-}$) are highly sensitive to PI, it is possible that both loss of NHEJ and NHEJ overactivity are required to some degree for PI sensitivity. Indeed, deficiency of DNA-pK, another component of NHEJ has also been shown to be required for PI sensitivity.[9] Our data propose that chromosomal instability disorders that have DNA repair defects and increased propensity to transform to leukemia and other cancers are potential targets for PI therapy. Furthermore, the identification of mutations in other key DNA repair genes such as the newly described oncogene *tip60*[22] increases the therapeutic potential of PI in a wide range of cancers.

## Authorship and Disclosures

TG: designed research, performed and analyzed data and wrote paper; SS, FF: designed research and analyzed data; GM: designed research and analyzed data and contributed to the writing of the manuscript.

The authors reported no potential conflicts of interest.



## References


1. Taniguchi T, D'Andrea AD. Molecular pathogenesis of Fanconi anemia: recent progress. Blood 2006; 107:4223-33.
2. German J. Bloom's syndrome. XX. The first 100 cancers. Cancer Genet Cytogenet 1997;93:100-6.
3. Stankovic T, Weber P, Stewart G, Bedenham T, Murray J, Byrd P, et al. Inactivation of ataxia telangiectasia mutated gene in B-cell chronic lymphocytic leukaemia. The Lancet 1999;353:26-9.
4. Varon R, Reis A, Henze G, von Einsiedel HG, Sperling K, Seeger K. Mutations in the Nijmegen Breakage Syndrome gene (NBS1) in childhood acute lymphoblastic leukemia (ALL). Canc Res 2001;61:3570-2.
5. Riballo E, Critchlow SE, Teo S-H, Doherty AJ, Priestley A, Broughton B, et al. Identification of a defect in DNA ligase IV in a radiosensitive leukaemia patient. Curr Biol 1999;9: 699-704.
6. Virag S, Szabo C. The therapeutic potential of Poly (ADP-ribose) Polymerase inhibitors. Pharm Rev 2002;54:375-429.
7. Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature 2005;434:913-7.
8. Farmer H, McCabe N, Lord CJ, Tutt ANJ, Johnson DA, Knights C, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature 2005;434:917-21.
9. Bryant HE, Helleday T. Inhibition of poly (ADP-ribose) polymerase activates ATM which is required for subsequent homologous recombination repair. Nucl Acid Res 2006;34: 1685-91.
10. Gaymes TJ, North PS, Brady N, Hickson ID, Mufti GJ, Rassool FV. Increased error-prone non homologous DNA End-Joining - A proposed mechanism of Chromosomal instability in Bloom's syndrome. Oncogene 2002;21:2525-33.
11. Gaymes TJ, Orr S, Mufti GJ, Rassool FV. Histone deacetylase inhibitors (HDI) induce DNA damage and repair: a mechanism for HDI dependent apoptosis in leukemia cells. Mol Cancer Res 2006;4:563-73.
12. Brady N, Gaymes TJ, Cheung M, Mufti GJ, Rassool FV. Increased error-prone NHEJ activity in myeloid leukemias is associated with DNA damage at sites that recruit key nonhomologous end-joining proteins. Cancer Res 2003;63:1798-805.
13. Tischkowitz MD, Morgan NV, Grimwade D, Eddy C, Ball S, Vorechovsky I, et al. Deletion and reduced expression of the Fanconi anemia FANCA gene in sporadic acute myeloid leukemia. Leukemia 2004;18:420-5.
14. Condie A, Powles RL, Hudson CD, Shepherd V, Bevan S, Yuille MR, et al. Analysis of the Fanconi anaemia complentation group A gene in acute myeloid leukemia. Leuk Lymphoma 2002;43:1849-53.
15. Yamamoto K, Hirano S, Ishiai M, Morishima K, Kitao H, Namikoshi K, et al. Fanconi Anemia protein FANCD2 promotes immunoglobulin gene conversion and DNA repair through a mechanism related to homologous recombination. Mol Cell Biol 2005;25:34-43
16. Nakanishi K, Yang Y-G, Pierce AJ, Taniguchi T, Digweed M, D'Andrea AD, et al. Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair. Proc Natl Acad Sci USA 2005;102:1110-5.
17 Taniguchi T, Garcia-Higuera I, Xu B, Andreassen PR, Gregory RC, Kim ST, et al. Convergence of the fanconi anemia and ataxia telangiectasia signaling pathways. Cell 2002;109:459-72.
18. Harmon FG, Kowalczykowski SC. RecQ helicase, in concert with RecA and SSB proteins, initiates and disrupts DNA recombination. Genes Dev 1998;12:1134-44.
19. Wu L, Hickson ID. The Bloom's syndrome helicase suppresses crossing over during homologous recombination. Nature 2003;18:870-4.
20. Chakraverty RK, Hickson ID. Defending genome integrity during DNA replication: a proposed role for RecQ family helicases. Bioessays 1999;21:286-94.
21. Donahue SL, Campbell C. A DNA double-strand break repair defect in Fanconi anemia. J Biol Chem 2002; 277:46243-7.
22. Gorrini C, Squatrito M, Luise C, Syed N, Perna D, Wark L, et al. Tip60 is a haplo-insufficient tumour suppressor required for an oncogene-induced DNA damage response. Nature 2007;448:1063-7.