DNAH2 facilitates the homologous recombination repair of Fanconi anemia pathway through modulating FANCD2 ubiquitination

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Abstract

Fanconi anemia (FA), an X-linked genetic or autosomal recessive disease, exhibits complicated pathogenesis. Previously, we detected the mutated Dynein Axonemal Heavy Chain 2 (*DNAH2*) gene in 2 FA cases. Herein, we further investigated the potential association between DNAH2 and the homologous recombination repair pathway of FA. The assays of homologous recombination repair, mitomycin C (MMC) sensitivity, immunofluorescence, and ubiquitination modification were performed in U2OS and DR-U2OS cell lines. In MMC-treated U2OS cells, the downregulation of the *DNAH2* gene increased the sensitivity of cells to DNA inter-strand crosslinks. We also observed the reduced enrichment of FANCD2 protein to DNA damage sites. Furthermore, the ubiquitination modification level of FANCD2 was influenced by the deficiency of DNAH2. Thus, our results suggest that *DNAH2* may modulate the cell homologous recombination repair partially by increasing the ubiquitination and the enrichment to DNA damage sites of FANCD2. *DNAH2* may act as a novel co-pathogenic gene of FA patients.

Keywords: DNAH2, FANCD2, Fanconi anemia, Homologous recombination, Ubiquitination

1. INTRODUCTION

Fanconi anemia (FA) is characterized by progressive bone marrow failure, congenital abnormalities, and cancer susceptibility.[1–5] Cells derived from FA patients display distinct patterns of chromosomal abnormalities.[1–6] In addition, these cells are hypersensitive to DNA crosslinking agents, such as mitomycin C (MMC).[7,8] There are more than 20 genes identified in the DNA repair pathway of FA.[9–13] However, the genetic and molecular basis of the diverse pathogenesis and clinical manifestations of FA remains elusive.

To gain insight into the pathogenesis underlying the clinical heterogeneity of FA, we previously performed whole-exome sequencing and subsequent Sanger sequencing verification analysis in 5 FA families.[14] We found that all 5 patients carried FANC gene mutations, and the genotypes of patients were FANCA (2 cases), FANCB (1 case), FANCM (1 case), and FANCD2 (1 case).[14] Interestingly, the mutations of *DNAH2* were identified in 2 FA cases.[14] One Dynein Axonemal Heavy Chain 2 (*DNAH2*) mutation, Chr17:7739411 A>T missense P>S, was in a FANCM mutated patient with stable condition.[14] The other *DNAH2* mutation, Chr17:7727684 A>T missense N>Y, was in a patient with FANCA mutation who has progressed to acute myeloid leukemia.[14]

Several studies reported that DNAH2 abnormalities might be linked to the presence of several human diseases. For instance, for the patients with multiple morphological abnormalities of flagella, DNAH2 deficiency in spermatozoa led to the severely disarranged axonemal structures with mitochondrial sheath defect.[15] *DNAH2* mutations were also observed in more than 10% of patients with chronic myelomonocytic leukemia.[16] But there is still a lack of investigation regarding the potential role of DNAH2 in FA and other hematological diseases.

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LC, XG, and YW contributed equally to this work.

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Author contributions: LC, CH, and MG performed the assays of cell culture, siRNA transfection, and RT-PCR. LC, XW, and CL performed the homologous recombination repair assay. LC, YW, WW, WA, and YW performed the MMC sensitivity and cell cycle assays. LC, YW, AZ, and YZ performed the western blotting analysis. LC, XG, and YW analyzed the genetic alteration of DNAH2 and drafted the manuscript. LC and XG revised the manuscript. WY and XZ performed the manuscript editing. All gave final approval and agreed to be accountable for all aspects of work ensuring integrity and accuracy.

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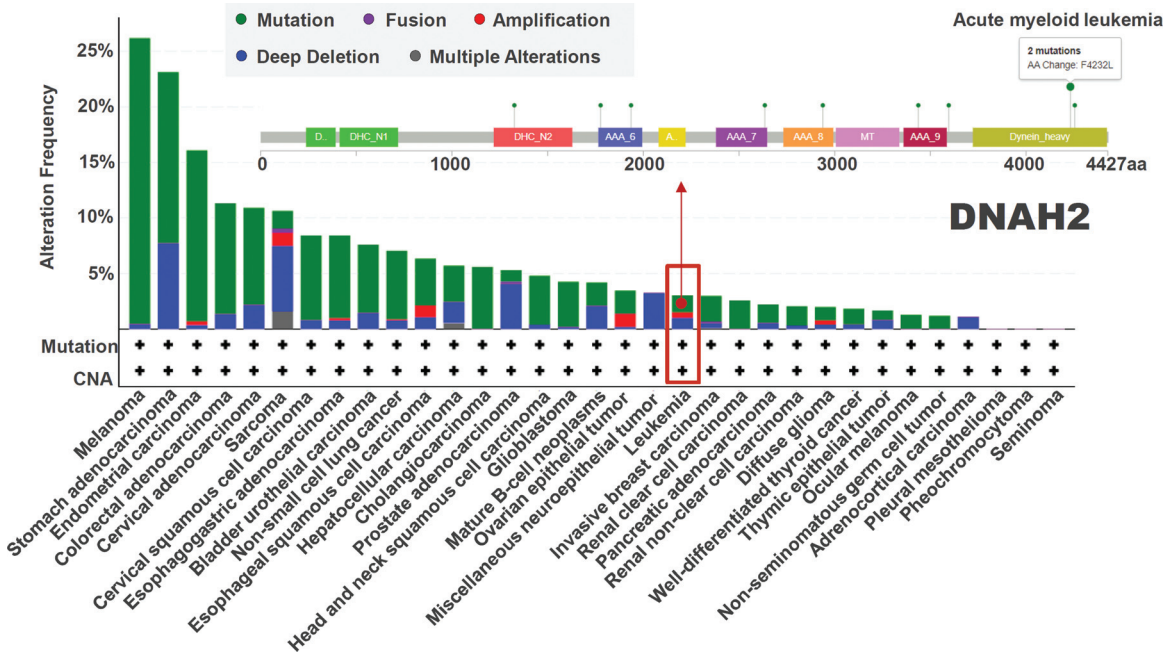

Figure 1. Genetic alteration of *DNAH2* for TCGA cancer cases. We utilized the cBioPortal to analyze the genetic alteration features of *DNAH2* for more than 30 types of cancers within the TCGA database, respectively. For leukemia, the specific mutated site information of DNAH2 was displayed in the schematic diagram of the DNAH2 protein structure. CAN: copy number alteration; *DNAH2*: Dynein Axonemal Heavy Chain 2.

In this study, we aim at studying the potential role of DNAH2 in the homologous recombination (HR) DNA repair pathway for FA cases. Our findings showed that DNAH2 was more likely to facilitate the homologous recombination repair of the FA pathway through modulating the ubiquitination modification and localization status of FANCD2 protein.

2. RESULTS

2.1. DNAH2 mutation occurs in a variety of tumor tissues

Given the link of FA and cancer,[17,18] we first utilized the cBioPortal web tool[19,20] to analyze the pattern and frequencies of *DNAH2* genetic alterations in a total of thirty-3 types of human cancers based on sequencing data within the TCGA database. The "Amplification," "Deep Deletion" of copy number alteration, "Mutation," "Fusion," and "Multiple Alterations" were detected. The data were visualized by a histogram. As shown in Fig. 1A, "Mutation" was the common type of *DNAH2* genetic alteration for most cancers, especially melanoma. With regards to leukemia cases, we observed both the "Mutation" and "Deep Deletion" of copy number of *DNAH2*. Furthermore, we analyzed the detailed mutation sites of *DNAH2* in the included leukemia cases but did not obtain the specific sites with high frequency (Fig. 1A, red arrow indication). The mutation of "F4232L" was only detected in 2 cases of acute myeloid leukemia (Fig. 1A). In addition, the previously identified 2 mutation sites in FA cases[14] were undetectable in the cancer cases of TCGA. Thus, the potential role of DNAH2 expression, but not genetic alteration, in the FA pathogenesis was focused in this study.

2.2. DNAH2 deficiency reduces cell viability of U2OS treated with MMC

Considering FA patients exhibit hypersensitivity to DNA interstrand crosslinks (ICLs),[7] we analyzed the potential effect of DNAH2 expression on the cellular responses to ICLs. We utilized the siRNA approach to down-regulate the expression level of *DNAH2* and *FANCA* (as positive control) in U2OS cell line. The mRNA level of *DNAH2* or *FANCA* was significantly decreased upon the transfection of DNAH2 siRNA (siDNAH2) or FANCA siRNA (siFANCA), when compared with siRNA control (siControl) (Fig. 2A). Then, we then detected the cell viability of U2OS under the treatment of MMC (an ICL-inducing agent) or not for 24 h. As shown in Fig. 2B, the reduced cell viability of U2OS was observed in the groups of siDNAH2, siFANCA, and siDNAH2+siFANCA, compared with the siControl group, at the different concentration points of MMC (0, 50, 100, 150, 200, and 250 ng/mL). Moreover, we found that the percentage of G2/M phase for U2OS cells was significantly increased upon the treatment of 100 ng/mL MMC in both siDNAH2 and siFANCA groups (Fig. 2C and D, $P < 0.001$), but not siControl group. These suggest that U2OS cells with DNAH2 deficiency exhibit the arrested cell cycle and the relatively weak resistance to MMC damage. DNAH2 maybe act as a FANCA-like factor to safeguard cell survival.

2.3. DNAH2 deficiency affects ubiquitination and nuclear localization of FANCD2 in MMC-treated U2OS cells

FANCD2 ubiquitination and localization to the impaired DNA loci are essential for the ICL repair event.[7] To further measure the influence of DNAH2 expression in the ubiquitination modification of FANCD2 protein in cells with ICL, we treated the U2OS cells of siControl, siDNAH2, and siFANCA, by MMC, respectively. Then, the ubiquitination level of FANCD2 was detected. As shown in Fig. 3A, we observed the knockdown of DNAH2 protein (lane 3–4, the second panel) and FANCA protein (lane 5–6, the third panel). The treatment of MMC could result in the presence of Ub-FANCD2 band signal in the siControl group (Fig. 3A, lane 2, the first panel). Nevertheless, the band

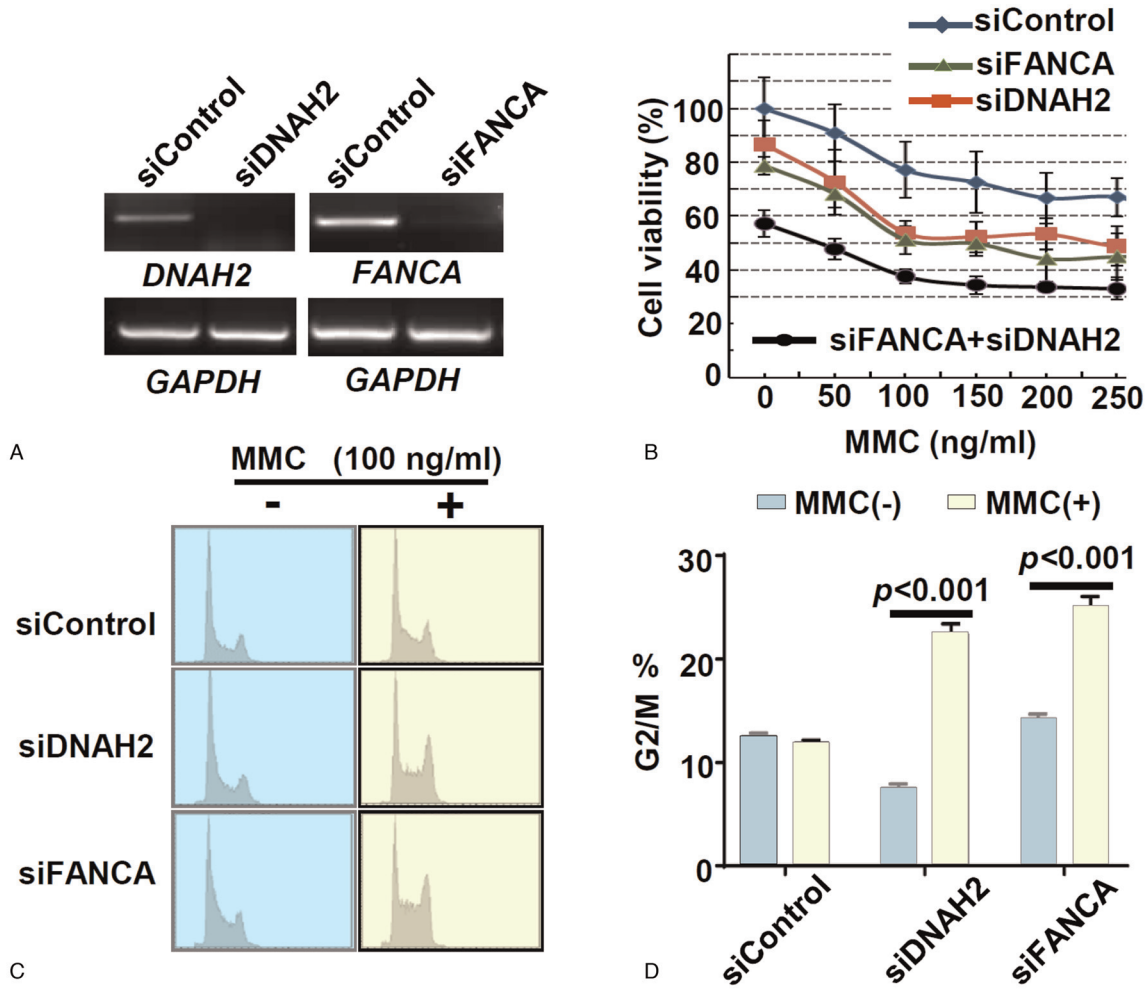

Figure 2. The effect of DNAH2 knockdown on the viability of MMC-treated U2OS cells. (A) The U2OS cell line was transfected with the siDNAH2 or siFANCA, respectively. The siControl was utilized as the control. The RT-PCR assay was performed to measure the expression level of *DNAH2* and *FANCA*, respectively. *GAPDH* gene was loaded as an internal reference gene. (B) The U2OS cell line was transfected with the siControl, siDNAH2, or siFANCA, respectively. Under the treatment of different concentrations of MMC (0, 50, 100, 150, 200, and 250 ng/mL), we measured the U2OS cell viability in the 3 groups, respectively. (C) and (D) In the presence (+) or absence (−) of 100 ng/mL MMC, we detected the status of the cell cycle and calculated the percentage of G2/M phase. The statistical difference between the MHC (−) and MHC (+) was analyzed by student *t* test. *DNAH2*, Dynein Axonemal Heavy Chain 2; MMC: mitomycin C; RT-PCR: reverse transcription-polymerase chain reaction.

signal disappeared in the groups of siDNAH2 (Fig. 3A, lane 4), and siFANCA (lane 6).

Next, we performed an immunofluorescence assay to analyze the potential impact of DNAH2 expression in the recruitment of FANCD2 protein into the ICL loci of U2OS cells. The γ-H2AX protein, a marker for HR repair, was used. As shown in Fig. 3B, DNAH2 deficiency failed to remarkably change the fluorescence intensity of FANCD2 and γ-H2AX within the nucleus of U2OS cells in the absence of MMC. We observed an enhanced nuclear fusion fluorescence signal of both FANCD2 and γ-H2AX in siControl group, when cells were treated by MMC (Fig. 3B). However, the fusion fluorescence signal was reduced in the siDNAH2 or siFANCA group (Fig. 3B). To quantitatively assess the influence of DNAH2 or FANCA on the activation of FA pathway and HR repair, we calculated the proportion of positive cells, which containing the fusion signal foci number of FANCD2 and γ-H2AX larger than 5, compared with siControl group, there was a statistically reduced percentage of positive cells (>5 foci) in both siDNAH2 and siFANCA groups (Fig. 3C, $P < 0.001$). These suggest the involvement of DNAH2 in the regulation of ubiquitination modification and nuclear localization of FANCD2 upon the DNA damage.

2.4. DNAH2 takes part in DNA HR repair process of U2OS

FANCD2, an integral component of the FA network, plays an essential role in the homologous recombination of chromosomal breaks.[21,22] Considering the inhibition effect of DNAH2 deficiency on the FANCD2 ubiquitination and localization, we tried to analyze the impact of DNAH2 on DNA HR repair. I-SceI/GFP (green fluorescent protein) reporter system was utilized.[23–25] Briefly, the presence of I-SceI cleavage site with a stop codon leads to the inactive state of SceGFP (Fig. 4A, red box). The expression of I-SceI mediated the cleavage of SceGFP and the presence of chromosomal double-strand breaks (DSB); and then, the cellular HR repair system involving multiple factors leads to the restoration of functional GFP cassette expression (Fig. 4A). The HR repair efficiency was evaluated by calculating the proportion of positive cells with GFP signals. For the U2OS of

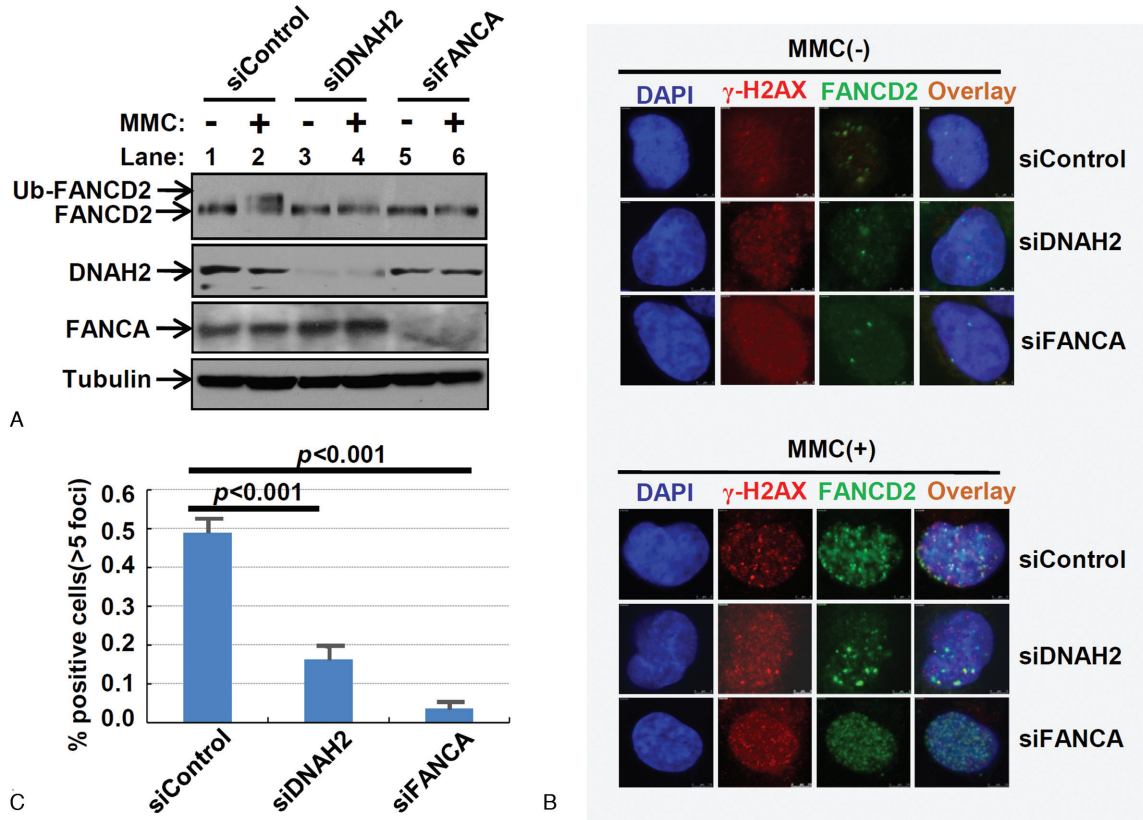

Figure 3. DHAH2 knockdown affects ubiquitination and nuclear localization of FANCD2. (A) U2OS cell line was transfected with the siControl, siDNAH2, or siFANCA, respectively. Under the treatment of MMC for 16 h (+) or not (−), a Western blotting assay was performed using the antibody specific for the FANCD2, DNAH2, FANCA, or Tubulin. (B) An immunofluorescence assay was also performed using the antibody specific for the γ-H2AX and FANCD2. (C) The positive cell rate (>5 foci) was measured, and the differences among the groups of siControl, siDNAH2, and siFANCA were analyzed using ANOVA followed by the LSD test. ANOVA: analysis of variance; *DNAH2*, Dynein Axonemal Heavy Chain 2; LSD: least significant difference; MMC: mitomycin C.

siControl, compared with I-Scel (−) group, there was an increased percentage of positive cells with GFP signals in the I-Scel (+) group, suggesting the successful HR-directed repair of DBS. The knockdown of both DNAH2 and FANCA statistically reduced the proportion of cells with GFP signal (Fig. 4B and C $P < 0.001$). These indicate that DNAH2 may be implicated in the HR repair process of chromosomal DSBs.

3. DISCUSSION

FA is one of the inherited human diseases with a rare incidence but very high risk of cancer-predisposition.[17,18] FA has been known to be caused by gene mutations in the FA pathway proteins that are involved in DNA repair and genome stability maintenance.[9–13] However, how FA patients develop diverse clinical manifestations of FA and/or progression to myelodysplastic syndromes and acute myelocytic leukemia? Previously, we observed the presence of *DNAH2* mutations in 2 FA patients. In the present study, we further analyzed the genetic alteration of *DNAH2* in more than thirty types of human cancers of TCGA and found that *DNAH2* is commonly mutated in most cancers. No specific mutation sites with high frequency were identified. For the acute myeloid leukemia cases, the "F4232L" mutation with the highest altered frequency was only detected in 2 cases. Unfortunately, we did not find the 2 mutation sites of *DNAH2* in previously detected FA cases.[14] Still, we cannot rule out the potential role of *DNAH2* mutation in FA pathogenesis. Thus, we focused on investigating DNAH2 expression in the cell viability, cell cycle, and homologous recombination repair pathway of FA.

We found that the downregulation of DNAH2 expression enhanced the sensitivity of U2OS cell line to MMC treatment. Furthermore, DNAH2 deficiency decreased the nuclear localization and ubiquitination of FANCD2 in U2OS treated with MMC, which resulted in a low level of DNA homologous recombination repair in U2OS cells. These support the idea that additional non-FA-pathway proteins, such as DNAH2, may also play important roles in FA pathogenesis. Our findings shed new light on the function of DNAH2 with FANCD2 in the FA pathway. DNAH2 may work as a synergistic pathogenic factor in the FA pathway. However, the deeper molecular mechanism of how DNAH2 regulates the FA pathway and affects FA pathogenesis still merits to be elucidated further.

DNAH2 was reported to be linked to the multiple morphological abnormalities of the sperm flagella.[15] DNAH2 contains a ring of 6 AAA-ATPase domains and 4 extensions.[26,27] It is worth mentioning that the above-mentioned 2 mutation sites of *DNAH2* are positioned at the P-loop NTPase structure of AAA2 in the second region and the third region, which may affect the interaction of DNAH2 with other proteins. More sample sizes are required to comprehensively analyze the mutation profile of *DNAH2* for FA patients and study the potential function links of FA and leukemia. The relationship of genetic alteration and expression level of *DNAH2* cause for our attention as well.

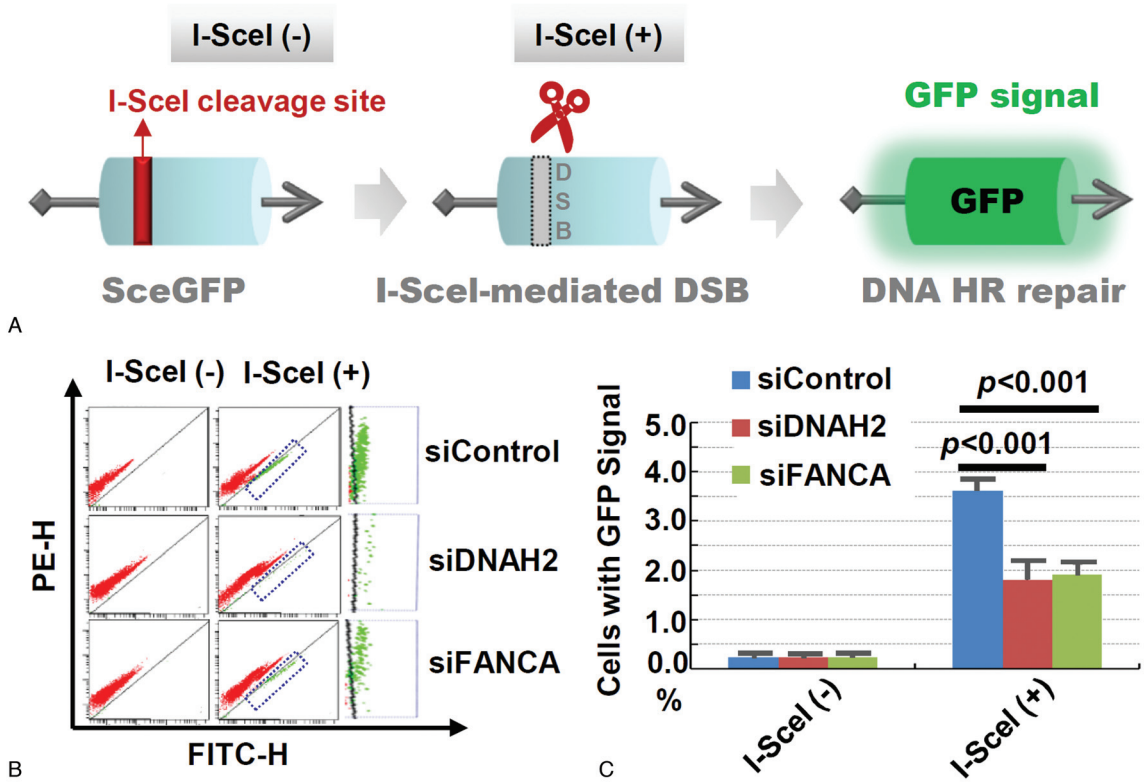

Figure 4. DHAH2 knockdown impairs DNA HR repair of U2OS cells. (A) DR-U2OS cell line with the I-SceI expression vector were used for the HR assay. The schematic diagram of the I-SceI/GFP reporter system was displayed. (B) DR-U2OS cell line was transfected with the siControl, siDNAH2, or siFANCA, respectively. the GFP signal was detected by a flow cytometric analysis. (C) The proportion of positive cells with GFP signals was calculated, respectively. The ANOVA followed by the LSD test was then performed. ANOVA: analysis of variance; *DNAH2*, Dynein Axonemal Heavy Chain 2; GFP: green fluorescent protein; HR: homologous recombination; LSD: least significant difference.

In summary, we explored the potential effect of DNAH2 in the homologous recombination repair of the FA pathway. DNAH2 expression might influence the sensitivity of cells to DNA interstrand crosslinks, and the ubiquitination modification and the recruitment of FANCD2 to DNA damage sites, which contributed to the involvement of DNAH2 in the HR repair pathway of FA. More evidence is required to come to light the potential mechanism of *DNAH2* mutation in the FA pathogenesis.

4. MATERIAL AND METHODS

4.1. Genetic alteration analysis of *DNAH2* in cancer cases of TCGA

Based on the cBioPortal database (https://www.cbioportal.org/), we analyzed the genetic features of *DNAH2* in the cancer patients of the TCGA project. The results of the alteration frequency, mutation type, and copy number changes were obtained by a "Cancer Types Summary" module. The mutated site information was also analyzed in the schematic diagram of DNAH2 protein structure by a "Mutations" module.

4.2. Cell culture and siRNA transfection

All cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 15% FBS (Biological Industries) and penicillin (50 U/mL)/streptomycin (50 μg/mL), at 37°C in a 5% CO_2 water jacket incubator (Thermo Scientific). The siRNAs were transfected using Lipofectamine RNAi MAX and Lipofectamine^{TM}2000 Reagents (Invitrogen; Opti-MEM, GIBCO), according to the manufacturer's protocol. The siRNA sequences: FANCA siRNA (siFANCA) [forward 5′-GGGTCAAGAGGGAAAAATA-3′; reverse 5′-GTGGCATCTTCACGTACAA-3′]; DNAH2 siRNA (siDNAH2) [forward 5′-GGAGUCGCUUCAACAUUAUTT-3′; reverse 5′-AUAAUGUUGAAGCGACUCCTT-3′]; negative control siRNA (siControl) [forward 5′-UUCUCCGAACGUGUCACGUTT-3′; reverse 5′-ACGUGACACGUU CGGAGAATT-3′].

4.3. Reverse transcription-polymerase chain reaction assay

Total RNA was extracted using the RNeasy Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized with an oligo-(dT) primer based on the manufacturer's instruction (TransScript First-Strand cDNA Synthesis SuperMix, TRANS). The mRNA expression was determined by reverse transcription-polymerase chain reaction (RT-PCR) (Applied Biosystems). The primer sequences: *DNAH2* [forward: 5′-TGGGCCAGTCCCGAAACCCA-3′; reverse: 5′-TGACGAGCCGGCCCGAGTTA-3′]; *GAPDH* [forward: 5′-CAAGGTCATCCATGACAACTTTG-3′; reverse: 5′-GTCCAC CACCCTGTTG CTGTAG-3′]; *FANCA* [forward: 5'-ATGGTCACTGCGTTCCTGGTT-3′; reverse: 5′-GGATGTTCCCCGTATGCTCAA-3′].

4.4. MMC sensitivity assay

U2OS cell line was transfected with the siControl, siDNAH2, or siFANCA, respectively, and then treated by the different concentrations of MMC (0, 50, 100, 150, 200, 250 ng/mL in

ddH_2O, Sigma) for 24 h. After the adding of CellTiter 96 Aqueous One Solution Reagent (Promega) for 2 h, cellular viability was measured using an UltraMark Microplate Imaging System (Bio Rad, Hercules, CA) following the manufacturer's protocol. In addition, under the treatment of 100 ng/mL MMC or not, a flow cytometric analysis was performed to analyze the cell cycle status, according to the manufacturer's protocol (BD FACS Aria TM cell sorter). The percentage of the G2/M phase was calculated, respectively.

4.5. Immunofluorescence assay

U2OS cells were grown on coverslips at low density (5×10^4 cells/mL) in 6-well plates and transfected with the siControl, siDNAH2, or siFANCA, respectively. After the treatment of MMC (80 ng/mL in ddH_2O, Sigma) or ddH_2O for 24 h, cells were fixed with 4% PFA in PBS for 10 min and permeabilized with 0.1% Triton X-100 and 0.5% NP-40 in PBS for 15 min at room temperature. Cells were incubated with primary antibody overnight at 4°C, the secondary antibody for 1 hour, and DAPI (Vector H-1200) dihydrochloride for 10 min at room temperature. The images were obtained using a confocal microscope (Leica TCS SP5). The primary antibody: mouse anti-FANCD2 (FI17, sc-20022, Santa Cruz), mouse anti-γH2AX (ab18311, Abcam). The secondary antibody: goat anti-mouse IgG-FITC (Invitrogen 62-6312), goat anti-mouse IgG-CY3 (EarthOx E031610).

4.6. Western blotting analysis

Western blotting assay was performed to detect the ubiquitination modification of FACND2, and the protein levels of FANCD2, DHAH2, and Tubulin. The U2OS cell line was transfected with the siControl, siDNAH2, or siFANCA, respectively. The cells were then treated or untreated with MMC for 16 h. About 50 μg protein lysate from each sample was extracted and denatured for separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the subsequent Western blotting assay. The primary antibodies: mouse anti-FANCD2 (FI17, sc-20022, Santa Cruz), mouse anti-DNAH2 (ab123683, Abcam), mouse anti-β-actin (AC-15, sc-69879, Santa Cruz), and mouse anti- FANCA antibody (C-20, sc-18664, Santa Cruz). The HRP-anti-mouse IgG antibody (KPL) was used as the secondary antibody.

4.7. Homologous recombination repair assay

DR-U2OS cell line was kindly provided by professor Yungui Yang (Beijing Institute of Genomics, Chinese Academy of Sciences). DR-U2OS cell line carries the DNA double-strand breaks substrate plasmid (DR-GFP), which can be recognized by I-SceI and utilized in homologous recombination repair assay. DR-U2OS cell was transfected with the siControl, siDNAH2, or siFANCA, respectively. Under the transfection of I-Scel expression plasmid (pCBASce), the GFP signal was detected by a flow cytometric analysis, according to the manufacturer's protocol (BD FACS Aria TM cell sorter). The proportion of positive cells with GFP signals was calculated.

4.8. Statistical analysis

Based on the SPSS12.0 software package (SPSS, Chicago, IL, USA), student's *t* test and one-way analysis of variance followed by least significant difference test were conducted. A two-tailed *P* value less than 0.05 was considered statistically significant.

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