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Original Article

A novel BRCA1 germline mutation promotes triple-negative breast cancer cells progression and enhances sensitivity to DNA damage agents

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ABSTRACT

Breast cancer is the most frequent malignancy and the second leading cause of cancer death in female worldwide. Compared with general population, patients with mutations in BRCA1 and BRCA2 genes confer approximately 10-fold increased risk of breast cancer. In this study, we conducted whole-exome sequencing to identify the disease-associated genes in a specific pedigree, in which at least eight individuals were diagnosed with cancers, including breast cancer, urothelial cancer, uterine cancer and colorectal cancer. Furthermore, a nonsense mutation *BRCA1* p.Trp372X was identified in the proband. The Sanger sequencing data has validated the same nonsense mutation in other 4 cancer patients and 3 normal family members. Additionally, functional experiments detected that this mutation was implicated in TNBC progression, manifesting as increased cell proliferation and migration. Cells with this mutation displayed impaired recruitment of RAD51 foci and unrepaired DNA damage, potentiating drug sensitivity to PARP inhibitor and cisplatin, both in the settings of combination use or monotherapy. On the basis of its occurrence in hereditary breast cancer and its identification in pedigree, as well as its function as a disruption of BRCA1, this mutation is critical to breast cancer predisposition and progression. Patients carrying this mutation may benefit from DNA damaging treatment regimens. Conclusively, we firstly reported this nonsense mutation in family pedigree and validated its pathogenicity through *in vitro* functional experiments.

Introduction

Remarkable advances in understanding breast cancer predisposition have been achieved in the last decade of twentieth century through the identification of two high-penetrance genes, *BRCA1* and *BRCA2* [1,2]. These two genes confer approximately 10-fold increased risk of breast cancer on population with such mutations compared to general population [3]. Deleterious mutations in *BRCA1* and *BRCA2* lead to inactivation of the encoded proteins, consequently failing to induce homologous recombination (HR) to repair the damaged DNA and maintain normal growth of cells. Germline loss-of-function mutations in BRCA1 or BRCA2 act as a threat for normal people, which increased the risk of breast cancer, as well as other malignancies, such as ovarian, stomach, pancreatic, prostate cancer. Patients with these loss-of-function mutations displayed increased risk of developing breast cancer with an estimated cumulative risk ranging from 36% to 71% at age 70 and up to 90% at age 80 [4] Genetic testing is vital as an important factor in the decision to undergo increased surveillance, prophylactic surgery or regimens' selection.

The BRCA1 gene is located on 17q2.1, cloned in 1994 with 22 exons. Its cDNA is 5589 bp and codes for a protein of 1863 amino acids, which comprises two important protein interaction domains including a zinc-binding RING domain at the N terminus and BRCT domains at the C terminus. The RING domain forms an enzymatically active ubiquitin-protein isopeptide ligase (E3) when BRCA1 heterodimerizes with BARD1 through its N terminus [5] BRCA1 protein could bind in a BRCT-dependent manner to several other proteins including p53, CtIP, and BACH1. The BRCT domain also cooperates with the RNA polymerase II holoenzyme to activate gene transcription [6] The BRCA2 gene is located at 13q12.3, and contains 28 exons. Its cDNA is 10,254 bp, coding for a protein of 3418 amino acids, which comprises multiple structure domains.

Abbreviations: BIC, Breast Cancer Information Core; DSBs, Double strand damages; HER-2, Human epidermal growth factor receptor 2; HR, Homologous recombination; TNBC, Triple negative breast cancer.

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Whole-exome sequencing (WES), an efficient and cost-effective DNA sequencing technology that screening multiple genes at the same time can provide a further insight into the etiology of breast cancer, and recognize the suspicious deleterious variants, based on the theory that disease-causing variants are rare and commonly locate in the protein-coding areas [7] Herein, to seek the potential genetic variants, we performed WES on the proband from a specific pedigree, in which at least eight individuals were diagnosed with cancers, including breast cancer, urothelial cancer, uterine cancer and colorectal cancer.

Materials and methods

Cases and clinical assessment

Detailed interviews were conducted with family members to collect information on pedigree structure, onset of disease, clinical and pathological information. 14 members of a four-generation family were genotyped. Informed consents were obtained from all participants, and the study was approved by the Institutional Review Board of Jinling Hospital (Nanjing, China).

Whole-exome sequencing and data analysis

Genomic DNA extracted from whole blood samples was enriched for exonic regions using Agilent liquid capture system (Agilent SureSelect Human All Exon V5). Libraries were sequenced on the Illumina Hiseq 4000 for paired-end 150 bp reads. Valid sequencing data is mapped to the reference genome (UCSC hg19) to get the original mapping. Further processing, including duplicate removal, local realignment and base quality recalibration, was performing using Picard and GATK. The SAMtools suite (http://samtools.sourceforge.net) was used to do variant calling and identify single-nucleotide variants (SNVs) and short insertions/deletions (INDELs). Variants were annotated with information such as the genome position and functional effect with the ANNOVAR tool (version 2014, November 12).

Variant filtering

Variants were filtered with the allelic frequency of 1% in the 1000 Genomes project (http://www.1000genomes.org), Exome Variant Server (http://evs.gs.washington.edu/EVS), Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org). Synonymous SNVs was discarded. Only variants predicted to change protein sequence (frameshift deletions and insertions, splicing alterations, and missense and nonsense SNVs) were considered. The retained nonsynonymous SNVs are submitted to PolyPhen (http://genetics.bwh.harvard.edu/ pph2), SIFT (Sorting Intolerant From Tolerant; http://sift.bii.astar.edu.sg), MutationTaster (http://www.mutationtaster.org), PhyloP (http://compgen.bscb.cornell.edu/phast/help-pages/phyloP.txt), GERP (Genomic Evolutionary Rate Profiling; http://mendel.stanford.edu/SidowLab/downloads/gerp), LRT (dbNSFP version 3.0) for deleterious prediction.

Variant validation and pedigree analysis

We confirmed the candidate causal variants by Sanger sequencing. The primers of the candidate variants were designed using Primer 3 (http://primer3.ut.ee/). Further, the c.1115C>*T* mutation in *BRCA1* was screen in 14 family members. During the PCR process, we used primers (forward primer: GGGAGGCTTGCCTTCTTCCG and reverse primer: TAGGCGGACTCCCAGCACAG). Sanger sequencing of PCR products was performed on Applied BiosystemsBigDye terminator sequencing chemistry and then run on an ABI3730xl genetic analyzer according to the manufacturer's instructions (Applied Biosystems, CA, USA). Sequence analysis was performed with Lasergene software (DNASTAR, Madison, WI, USA).

Cell lines culture, reagents and lentivirus transfection

Breast cancer cell lines MDA-MB-231, Hs578t, BT-20, MDA-MB-436, HCC1937, and MDA-MB-157 were purchased from the Chinese Academy of Science Committee type culture collection cell bank (Shanghai, China) during 2016–2017. Authenticity of these cell lines with STR DNA typing was achieved by cell bank. Cells were maintained in RPMI 1640, DMEM, or L-15 containing 10% FBS and 1% penicillin/streptomycin. Olaparib and ABT-888 were purchased from MedChem Express. Cisplatin and Paclitaxel were from Hansoh Pharmaceutical Co. (Jiangsu, China). To identify the deleterious function of *BRCA1* p.Trp372X, we transfected BRCA wild-type cells MDA-MB-231, Hs578t with recombinant lentivirus of *BRCA1* p.Trp372X ordered from GenePharma (Shanghai, China). The *BRCA1* p.Trp372X was produced and then confirmed by DNA sequencing. A nonspecific control was also purchased from GenePharma. Cells were harvested for further experiments after 72 h of transfection.

Cell survival assay

For cell viability analysis, cells were seeded in 96-well plates at 4000–6000 cells per well. On the following day, cells were exposed to different concentrations of agents and after 72 h exposure cell survival was assessed with the Cell Counting Kit-8 in accordance with the recommended guideline (MedChemExpress, Monmouth Junction, NJ, USA). Combination index (CI) values, calculated using CompuSyn software (ComboSyn, Inc., NJ, USA).

Colony formation assay

Cells were seeded in 6-well plates at a concentration of 500 cells in 2 ml of medium per well and cultured in medium for 12–14 days. After fixation, colonies were stained with 0.5% crystal violet for 30 min.

Cell migration assay

Cell migration was measured by transwell assay (Corning Incorporated, Corning, NY, USA) with 24-well uncoated transwell cell culture chambers. Cancer cells (2×10^4) cultured in serum-free medium (200 μL) were added to the upper chamber. The medium (800 μL) containing 10% FBS was added to the lower chamber. The cells in the upper chamber were carefully removed with a cotton swab after 24 h incubation. Cells on the lower chamber were fixed with 100% methanol for 30 min and then stained with 0.5% crystal violet for 15 min. The migrated cells were observed by inverted microscopy.

Immunofluorescence

Cells were harvested, and fixed in the 4% paraformaldehyde and consequently permeabilized with 0.5%tritonX-100. All Cells were incubated overnight at 4 °C with the primary antibodies [anti-RAD51 (abcam, ab133534) 1/800, or anti-γH2AX (Cell Signaling Technology, 20E3) 1/300]. Secondary Alexa Fluor 594 was used to immunoprecipitate the primary antibody. Finally, Coverslips were mounted with DAPI and visualized with a Zeiss Scope A1 fluorescence microscope. Cells were scored positive for RAD51 and γH2AX foci if more than 10 nuclear foci exist, and approximately 100 cells were scored.

Comet analysis

Cell suspension were harvested and mixed with 1.2% low melting agarose and the mixture was added over 1% agarose coated

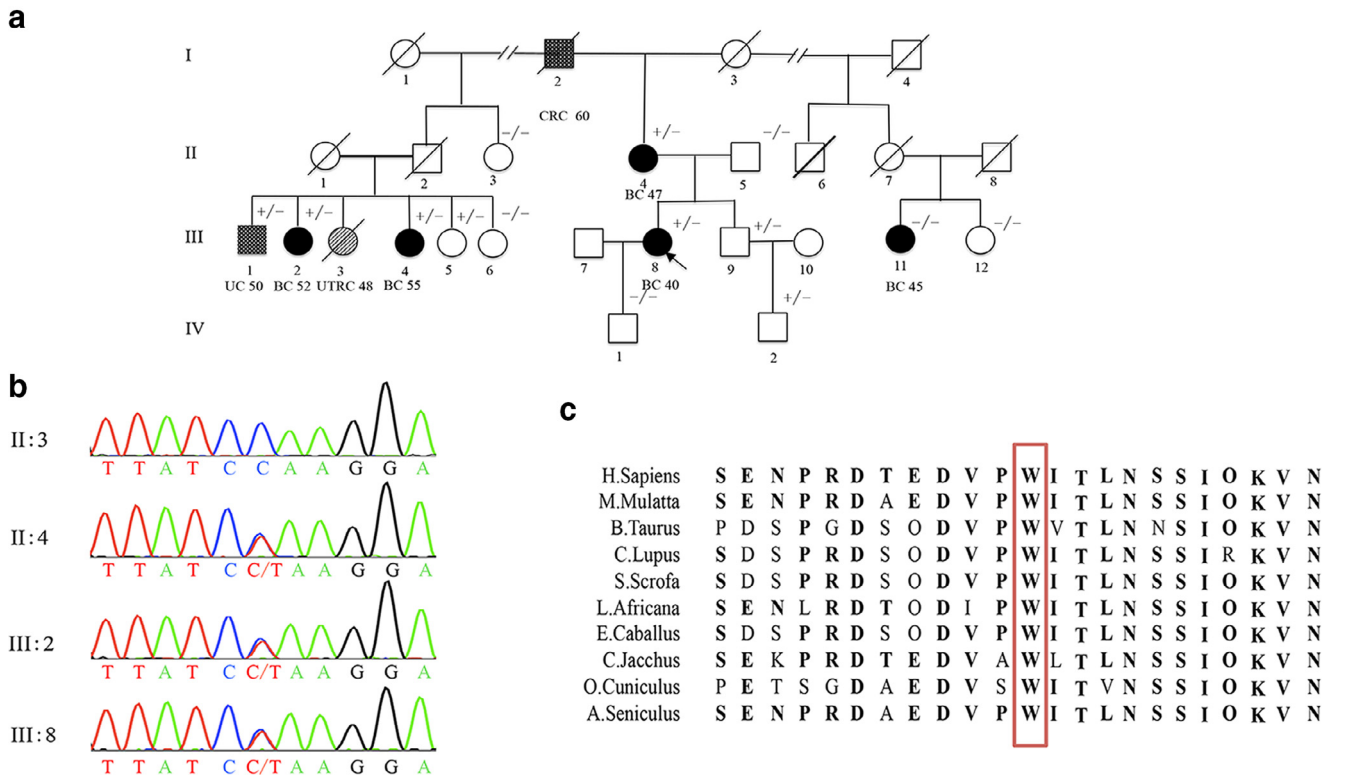

Fig. 1. Pedigree of *BRCA1* mutation carriers. The proband was indicated by an arrow. The proband was analyzed by whole-exome sequencing. Cancer type and age at diagnosis are indicated below cancer patients. Tested family members are marked with +/- for heterozygous mutation carriers and with -/- for wild-type. Single slush indicates a deceased individual. Double slush means that the family members rebuilt a new family after divorce. (**a**) Pedigree structure of the studied family. (**b**) Electropherograms of Sanger sequencing confirmed the *BRCA1* c.1115C>*T*, p.W372X variant. (**c**) The conservation of this variant among different species. BC, breast cancer; CRC, colorectal cancer; UTRC, uterine cancer; UC, urothelial carcinoma.

fully frosted slides (Thermo-Fischer Scientific). The slides were incubated in lysis buffer overnight at 4. Further, alkaline denaturation was carried out in an electrophoresis chamber for 20 min. Then we run the electrophoresis at 25 V and 300 mA for 20–25 min. The slides were stained with PI at dark for 5 min. Images were taken with a Zeiss Scope A1 fluorescence microscope. The quantification of tail DNA was measured by CASP software.

Statistical analysis

All experiments in cells were performed in triplicate. Statistical analysis was carried out using student's *t*-test using GraphPad Prism software (GraphPad Prism, San Diego). A P value of <0.05 was considered statistically significant.

Results

WES identified a nonsense mutation of BRCA1

The pedigree is presented in Fig. 1. The proband was diagnosed with triple negative breast cancer at the age of 40. In this pedigree, her mother was also diagnosed with a high-grade invasive breast cancer (estrogen receptor positive, progesterone receptor negative, and human epidermal growth factor receptor negative) at 47 years old. The proband's grandfather died of colorectal cancer. In addition, four half first cousins, who shared same grandfather with the proband, were affected with cancers. Among four first cousins, two were diagnosed with breast cancer, one with urothelial cancer, and another died of uterine cancer. Furthermore, one half first cousin, who shared the same grandmother, was diagnosed with breast cancer at 45 years old. In total, at least eight individuals in this family were diagnosed with cancers, including breast cancer, urothelial cancer, uterine cancer, and colorectal cancer. We performed WES analysis on the proband and detected a nonsense mutation in BRCA1, c.1115C>*T*. This change causes a premature stop codon at position 372 (p.Trp372X, also named as W372X), and therefore, we considered it to be pathogenic. Based on available DNA samples, the variant was further identified in this selected family. The proband and her mother, together with three half first cousins affected with cancers were positive for BRCA1 c.1115C>*T*, meanwhile, three healthy family members indicated in Fig. 1 were detected with the same stop-gain alteration. However, the half cousin, who shared the same grandmother with the proband, did not carry the mutation.

BRCA1 W372X promotes BRCA wild-type TNBC cells progression in vitro

Next, to evaluate the functional change associated with *BRCA1* W372X mutation, a series of functional experiments were implemented to identify the deleterious activity of this mutation. BRCA1 has been implicated in numerous cellular processes, and loss of BRCA1 has chromosomal abnormalities, G2/M checkpoint loss and DNA repair defects. Furthermore, to illustrate whether this mutation could trigger increased cell progression in triple negative subtype cancer cells, we transfected BRCA wild-type cell lines with the cloned W372X mutant. Cell viability assays showed that the *BRCA1* mutation increased cell proliferation in MDA-MB-231, Hs578t and BT-20 cells (Fig. 2a–c). Consistent with cell viability assays, colony formation assays confirmed that cells transfected with the cloned W372X showed higher colony formation compared with wild-type TNBC cells (Fig. 2e). Moreover, the W372X mutation promoted cell migration more significantly in BRCA1 W372X overexpressed cells compared with the wild-type cells (Fig. 2d).

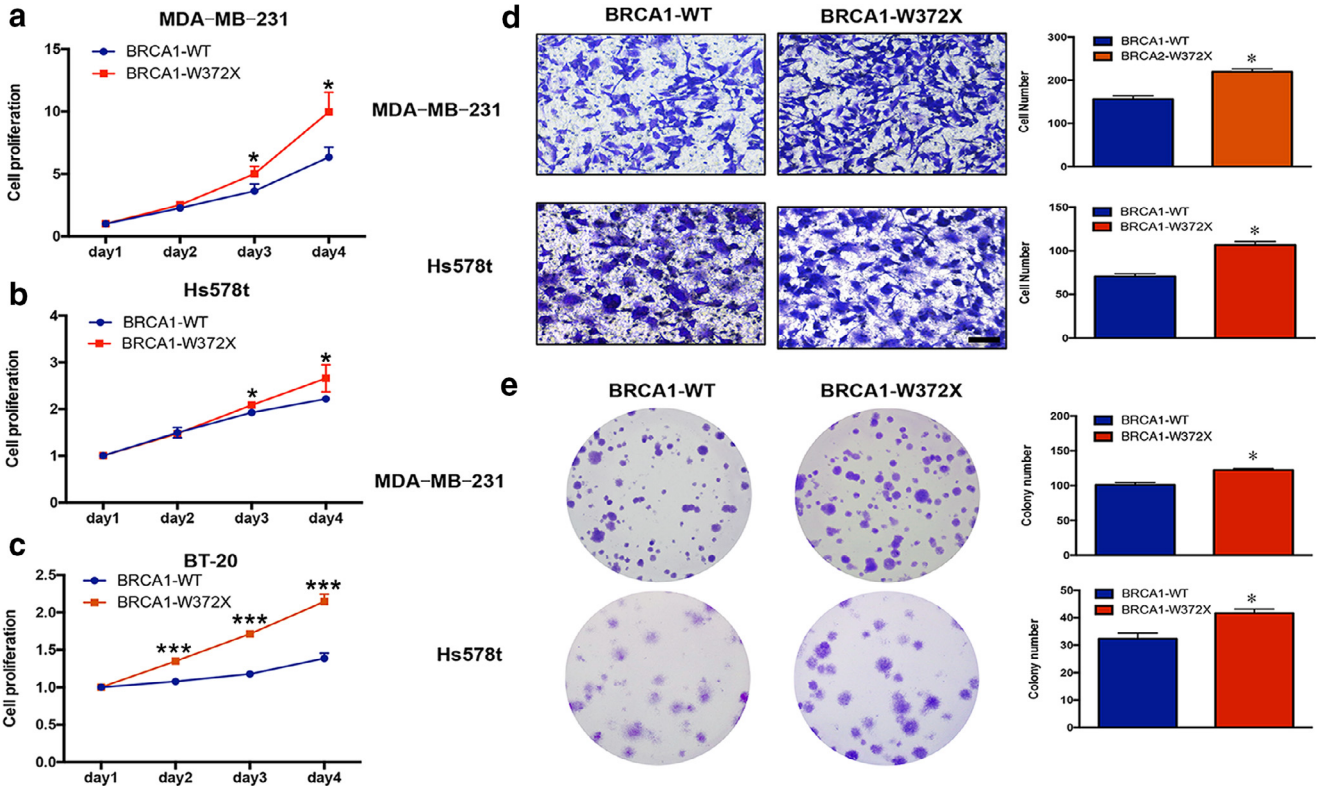

Fig. 2. The W372X mutation of *BRCA1* Promotes triple negative breast cancer progression *in vitro*. (**a-c**) Cell viability experiments for TNBC cells expressing *BRCA1* W372X or not, we conducted the experiments on MDA-MB-231 (**a**), Hs578t (**b**), and BT20 (**c**) cells. $^{*}p \leq 0.05$, $^{***}p \leq 0.001$. (**d**) Transwell experiment for the migration ability of MDA-MB-231 and Hs578t cells, cells on the bottom surface of the well were counted, $^{*}p \leq 0.05$. Error bars represent means ± SD. Scale bar, 50 um. (**e**) Colony formation assay for the growth of MDA-MB-231 and Hs578t cells, with or without *BRCA1* W372X transfection. The numbers of the colony formation were counted and compared with the control group, $^{*}p \leq 0.05$.

BRCA1 W372X sensitizes BRCA wild-type TNBC cells to DNA damage agents through impairing homologous recombination of BRCA1

Given the tight relationship between BRCA mutations and triple negative subtype, we further treated six TNBC cell lines, designated as BRCA wild type or BRCA mutant TNBC cell lines, with PARP inhibitors, cisplatin and paclitaxel respectively. We found that BRCA mutant TNBC cell lines, MDA-MB-436 and HCC1937, were preferentially sensitive to olaparib and cisplatin compared with BRCA wild-type TNBC cell lines Hs578t, MDA-MB-231, MDA-MB-157 and BT-20 cell lines (Fig. 3a and 3b). However, the sensitivity was not observed in cells treated with paclitaxel (Fig. 3c). The half-maximal inhibitory concentration (IC50) values following the treatment of cisplatin and olaparib was significantly lower in BRCA mutant cell lines (Fig. 3a and 3b). We speculated that BRCA mutations in TNBC cells could give rise to DNA repair defects to enable a better response after exposure to DNA damaging regimens, such as cisplatin and PARP inhibitors. For *BRCA1* W372X mutation, to establish whether it could also lead to DNA repair defects in carriers, we transfected BRCA wild-type TNBC cell lines with the cloned W372X mutant, and selected the successfully infected cells with puromycin. Of note, when cells were exposed to cisplatin or ABT-888, another PARP inhibitor, we found that cells expressing BRCA1 W372X exhibited a lower survival rate than wild-type TNBC cells, MDA-MB-231 cells in particular (Fig. 3d–f). What is more, the W372X mutation of *BRCA1* could also enhance synthetic lethality of combination use of ABT-888 and cisplatin in MDA-MB-231 and Hs578t cells (Fig. S1). These data suggest that this mutation significantly enhanced the sensitivity of BRCA wild-type TNBC cells to DNA damaging agents.

To explore the mechanism accounting for the sensitivity to DNA damaging agents, we measured the degree of DNA damage induced by cisplatin using a comet assay. As a result, enhanced DNA damage was detected in cells that expressed W372X mutants in response to cisplatin (Fig. 4a and 4b). Furthermore, we used the immunofluorescence staining to detect the formation of phosphorylated histone H2AX (γH2AX) foci in the cells after DNA damage exposure. Consistent with previous results, the numbers of γH2AX-positive foci were highly detected in cells with the expression of mutant W372X (Fig. 4c and 4d). Moreover, to test whether reduced homologous recombination (HR) was associated with enhanced cisplatin sensitivity in W372X mutant cells, we detected the foci formation of RAD51 following the DNA damage of cisplatin in MDA-MB-231 cells. Impaired HR by the decreased RAD51 foci formation indicated that *BRCA1* W372X could decrease the HR efficiency to enhance cisplatin sensitivity (Fig. 4e and 4f).

In sum, exome sequencing was performed on the proband from a specific family. This family has 8 family members affected with cancers and five of them were diagnosed with breast cancers. Functional experiments suggested that the *BRCA1* W372X mutation was deleterious which could enhance cancer progression in TNBC cells. Further, cells expressing the *BRCA1* W372X variant showed increased sensitivity to DNA damaging agents with impaired HR function. Briefly, this mutation was critical to TNBC progression, and patients with such a mutation might have a better prognosis if treated with DNA damaging regimens.

Discussion

In this study, our proband that displays the triple negative subtype carried the BRCA1 nonsense mutation. Compared to other subtypes, TNBC is associated with early recurrence of disease and poor outcome. Besides, germline mutations in *BRCA1* have been

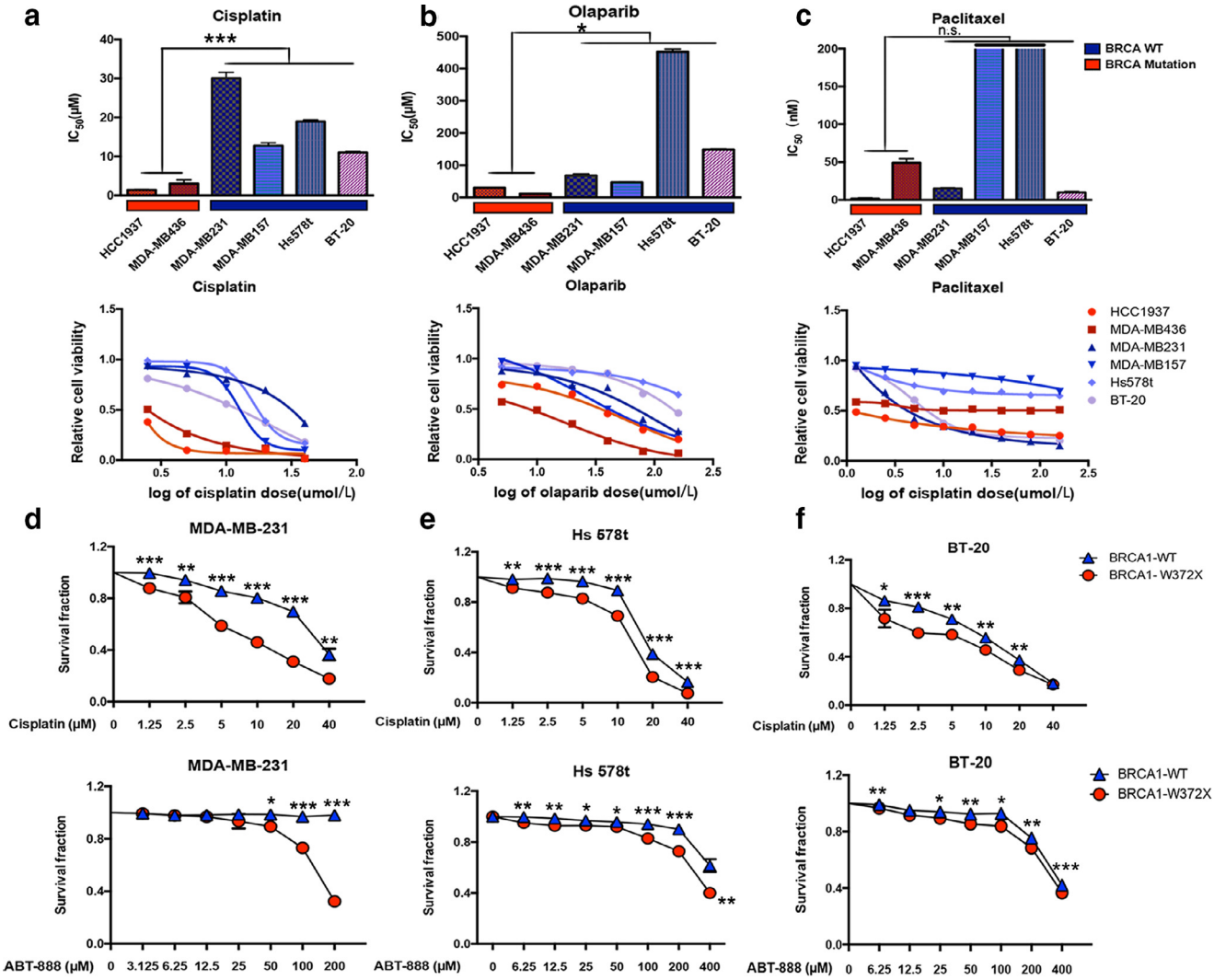

Fig. 3. BRCA1 W372X sensitizes BRCA wild-type TNBC cells to DNA damage agents. (**a–c**) IC50 values and cell growth curves for BRCA wild-type and mutant TNBC cells treated with cisplatin (**a**), Olaparib (**b**), and paclitaxel (**c**) for 72 h. Black horizontal lines above various bars indicated that IC50 was larger than the maximum value of Y-axis. Cell lines that carry BRCA mutations (left) or not (right) were illustrated below the graph. $^{*}p \leq 0.05$, $^{***}p \leq 0.001$, n.s., not significant. (**d–f**) *BRCA1* W372X increased the sensitivity of the MDA-MB-231 (**d**), Hs578t (**e**), and BT-20 cells (**f**) to the treatment of cisplatin or ABT-888, another PARP inhibitor. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

detected in up to 11–20% of TNBC, and TNBC accounts for 70% in breast cancer patients with *BRCA1* mutations [8] Couch et al. identified *BRCA1* mutations in up to 15.5% of TNBC patients with age at diagnosis < 40 years through panel-based screening in a large series of TNBC cases [9] Moreover, *BRCA1* mutations are associated with not only the risk of breast and ovarian cancer, but also the prognosis after the treatment of DNA damage agents. Given the essential role of BRCA1/2 in DNA repair of double strand damage (DSBs), tumors with BRCA mutation could display more sensitivity to DNA damaging agents [10] By grouping multiple TNBC cell lines to BRCA wild type and mutant classes, we validated that BRCA-mutated TNBC cells were more sensitive to cisplatin compared with BRCA wild-type TNBC, but no sensitivity was observed when treated with paclitaxel in these cells.

The previous studies have found that *BRCA1* mutations mainly occurred in three regions: N-terminal RING (exons 2–7), exons 11–13 (with multiple protein binding sites and functional domains), and C-terminus BRCT (exons 16–24) [11] Exon 11 was the most frequently mutated region such as c.A1069T, p.K357* and c.3419delGinsTGACTACTG, p.S1140fs, accounting for 60% of *BRCA1* mutations [12] Mutations in this region may disrupt the function of *BRCA1* protein involving in DNA repair, cell cycle, or nuclear localization [13] In this study, much attention was drawn on a specific family, in which multiple cancers occurred. Both grandparents of the proband set up new families after divorce. Among all members from the pedigree, eight individuals were diagnosed with cancers, including breast cancer, urothelial cancer, uterine cancer and colorectal cancer. By using whole-exome sequencing, we detected a nonsense mutation in *BRCA1* c.1115C>*T* in the proband, and further Sanger sequencing of other family members confirmed the deleterious role of this mutant. This variant was previously detected in an inherited breast cancer patient who was diagnosed at the age of 29 [14] In silico analysis showed that this variant was predicted to encode a truncated non-functional protein, while no available functional studies were conducted to characterize the effect of this variant. Previous study demonstrated that somatic loss of BRCA1 and P53 resulted in the rapid formation of more aggressive carcinoma [15] Consistent with this, our research discovered that the truncated variant of *BRCA1* could induce cancer progression in TNBC cancer cells [16] The deficiency of BRCA1 caused impaired DNA damage repair and cell growth arrest, while the inactivation of certain checkpoint gene, which highly occurred in TNBC, subsequently induced the escape of cell cycle checkpoints, leading to the uncontrolled cell proliferation and tumorigenesis.

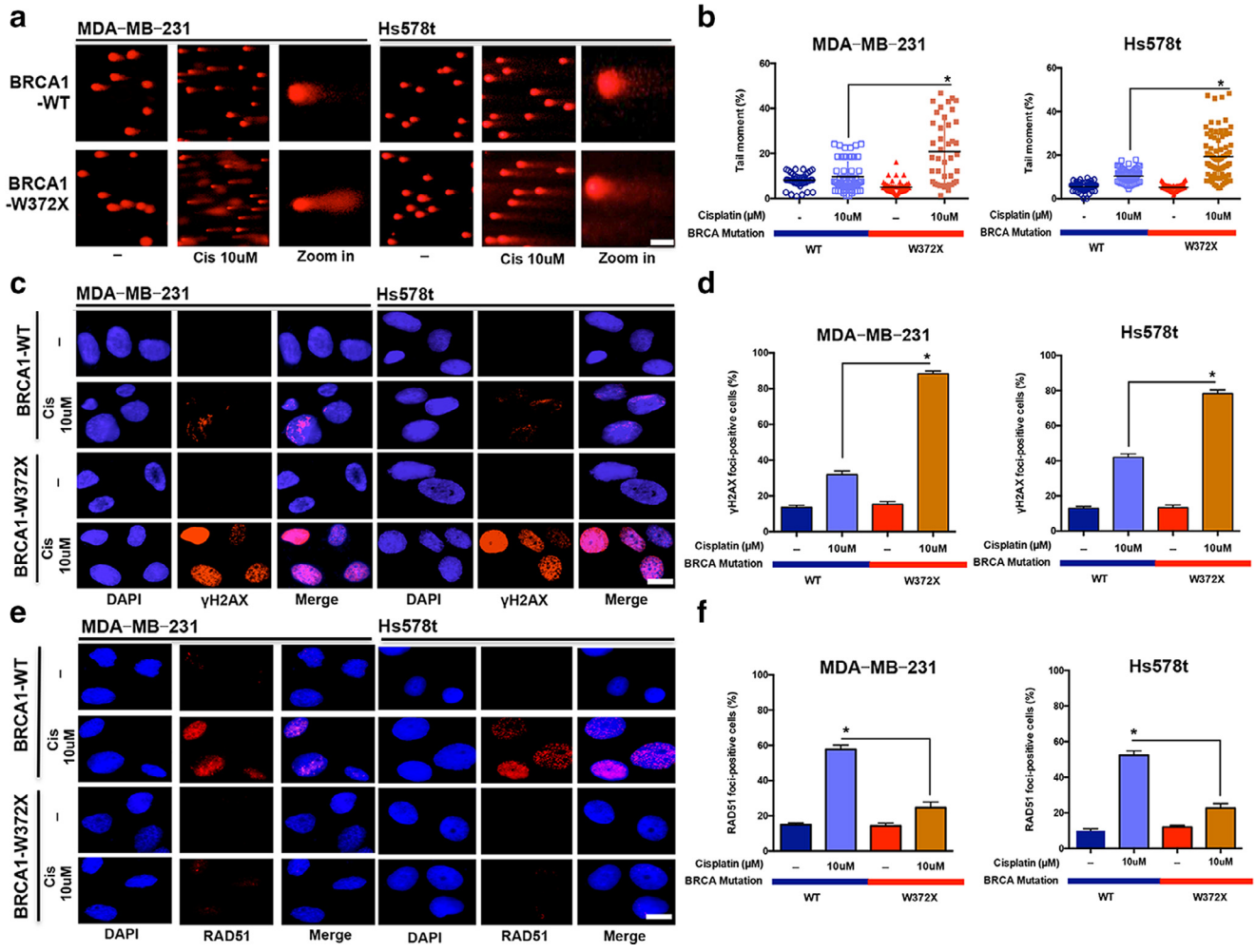

Fig. 4. *BRCA1* W372X increases the sensitivity of BRCA wild-type TNBC cells to DNA damage agents through impairing HR of BRCA1. (**a** and **b**) DNA damage in MDA-MB-231, Hs578t, treated with cisplatin, with or without *BRCA1* W372X transfection, measured by comet assays. The extent of DNA damage was quantified by the tail moment in the comet assay. Bars represent mean values of the tail moment. Scale bar, 10 um. (**c** and **d**) The immunofluorescence staining of phosphorylated histone H2AX (γH2AX) foci in the MDA-MB-231 and Hs578t cells, with or without *BRCA1* W372X transfection, after DNA damage exposure to cisplatin. Quantification of the number of foci was illustrated. Scale bar, 10um. (**e** and **f**) The immunofluorescence assay of the HR marker, RAD51, in MDA-MB-231 and Hs578t cells. At least 100 cells were counted. Cells were scored positive forγH2AX foci if more than 10 nuclear foci exist. $^{*}p \leq 0.05$. Scale bar, 10 um.

In summary, by performing WES on the proband from specific pedigree with multiple cancers, we identified a truncated variant of *BRCA1* W372X. Further pedigree analysis and functional studies identified that it was deleterious and this *BRCA1* variant was implicated in TNBC cancer progression, together with its enhancement of sensitivity to DNA damaging drugs, which were firstly identified in this study.

Declaration of Competing interest

The authors declare that they have no conflict of interest.

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Ethics approval and consent to participate

Informed consents were obtained from all participants, and the study was approved by the Ethics Committee of Jinling Hospital (Nanjing, China).

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Acknowledgments

Not applicable

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cancergen.2019.08.004.

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