Functional analysis of BRCT missense mutations in BRCA1-mutated Chinese Han familial breast cancer

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Abstract. Breast cancer 1 (BRCA1) is one of the most common tumor suppressor genes in breast cancer. The BRCT domain of BRCA1 has been shown to have a critical role in tumor suppression. In a previous study, two de novo BRCT missense mutations of BRCA1, G1763V and L1786P were identified from Chinese females with familial breast cancer. In the present study, the function of these two novel mutations were assessed by bioinformatics analysis and a series of experiments investigating cell proliferation, cell cycle and chemotherapy combination. Although bioinformatics analysis indicated that the mutants may be deleterious, a series of experiments revealed that the two mutants significantly reduced the growth and increased cell apoptosis similar to the function of BRCA1 wild type. Furthermore, no synergistic effect between the Olaparib and BRCA1 mutation was noted on cell apoptosis. These results demonstrated that these two mutations did not affect the tumor suppressor function of BRCA1. It was concluded that not all BRCA1 missense mutations are pathogenic and that any new BRCA1 mutation should be assessed for its effect on the tumor suppressor function of BRCA1.

Introduction

Breast cancer is one of the most common malignant neoplasms in women. In total, 5-10% of breast cancer cases are associated with genetic susceptibility, and the most common breast cancer susceptibility genes are breast cancer 1 (BRCA1) and breast cancer 2 (BRCA2) (1). Differences in mutations of BRCA1 and BRCA2 have been reported among different ethnic populations. Bergman et al (2) reported a familial mutation rate of up to 36% in BRCA1 or BRCA2 in Western Swedish breast cancer families. In Chinese females with familial breast cancer the mutation rate of BRCA1 and BRCA2 was ~10%, of which 50-60% of the mutations have not been previously reported, and their functions are unknown (3).

The BRCA1 gene is a tumor suppressor gene that is located on 17q21, with a total length of ~100 kb. BRCA1 consists of 24 exons and encodes a protein with 1,863 amino acids. BRCA1 has an important role in DNA damage repair, cell cycle control, protein ubiquitination and chromatin remodeling (4). BRCA1 includes two important functional domains, namely really interesting new gene (RING) domain in the N-terminal and BRCA1 C terminus (BRCT) domain in the C-terminal. Several studies have shown that the RING domain of BRCA1 interacts with BRCA1-associated RING domain protein 1 to form a powerful E3 ubiquitin ligase and has a role in tumor suppression by regulating several signaling transduction pathways (5,6). However, Shakya et al (7) reported that the BRCT domain was the structure with tumor suppressor function, since point mutations of the BRCT domain caused rapid formation of tumors in mouse models.

Over 500 different BRCA1 mutations have been identified throughout its coding region, including nonsense mutations, missense mutations, frame shift insertions or deletions, as well as mutations in the untranslated region (8). Among these mutations, frame shift insertions or deletions or nonsense mutations, resulting in 20-30% of familial breast cancer cases, are the most deleterious and usually result in the formation of a truncated protein. For example, the 185delAG BRCA1 mutation led to the loss of all known functional domains (9). Missense mutations, accounting for 5-10% of familial breast cancer cases, often occur in the coding region of the highly conserved amino acids (10). Missense mutations usually only partially affect the function of wild-type BRCA1 and rarely cause loss of the entire structure or function, while the clinical significance of these missense mutations is often uncertain (11). As a consequence, 10-20% of patients with familial breast cancer...
cannot receive any meaningful information from clinical genetic testing (8,11).

Two missense mutations, G1763V and L1786P were identified from Chinese females with familial breast cancer in our previous study (unpublished data). These two mutations, which are located in the BRCT domain, have not been previously described in the database established by the Breast Cancer Information Core. The present study investigated the function of these two mutations and revealed that these novel missense mutations did not affect the tumor suppressor function of the BRCA1 gene.

Materials and methods

Cell line and cell culture. The BRCA1-mutated breast cancer HCC1937 cell line was obtained from the Cell Bank of Shanghai Institute of Cell Biology (Shanghai, China). No wild-type BRCA protein was produced in the HCC1937 cell line. The cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere with 5% CO₂.

Construction of mutated plasmids and transfection. A plasmid containing a full-length BRCA1 cDNA on a pcDNA3.1 backbone was provided by Dr. Genze Shao (Peking University Health Science Center, Beijing, China), for the sub-cloning and generation of the mutated expression constructs. The G1763V and L1786P mutations were generated in this plasmid using QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA, USA). Primer pairs for the sub-cloning and generation of the mutated expression constructs were as follows: 1763 mutation forward, 5’-AGG ACA GAA AGA ATC TTTC TGT CCT GGG ATT CTC TTG-3’ and reverse, 5’-CCA CAG CAC ACg GCT GTA CCA TCC ATT CC-3’. Large-scale DNA preparations were made using the Qiagen Plasmid Miniprep Maxi Kit (Qiagen, Inc., Valencia, CA, USA). Each plasmid was sequenced entirely to verify their identity. For gene transfection, HCC1937 cells were grown overnight at 37°C with 5% humidified atmosphere with 5% CO₂.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from transfected HCC1937 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Reverse transcription was performed using a total of 1 μg RNA, oligo (dT) 15 primer, and the ThermoScript reverse transcription kit (Invitrogen; Thermo Fisher Scientific, Inc.). RT-qPCR was performed using SYBR Green PCR Master Mix (Thermo Fisher Scientific, Inc.) on an ABI7500 Real-Time PCR System (Thermo Fisher Scientific, Inc.). qPCR was performed using the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 30 sec and 60°C for 1 min. Primers for BRCA1, poly (ADP-ribose) polymerase 1 (PARP) and GAPDH were as follows: BRCA1_F, 5’-AGG TCCAAAGCGGCAAGG-3’ and BRCA1_R, 5’-AGGTTGC CTCACATCTGGC-3’; PARP_F, 5’-CCTAAGGGCTCA GAAGCACC-3’ and PARP_R, 5’-AGGAGGGCACCAGA ACC-3’; and GAPDH_F, 5’-AGGTTGGAGTCTACGGAT TG-3’ and GAPDH_R, 5’-GTGATGCGTGGACCTTGGA GT-3’. The results of BRCA1 and PARP were normalized to GAPDH. Data were calculated based on 2-ΔΔCq, where ΔCq=Cq (Target)-Cq (Reference). Fold change was calculated using the 2-ΔΔCq method (12).

Protein extraction and immunoblotting. Proteins were extracted from cells using radioimmunoprecipitation assay buffer containing complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Proteins were separated using 8% SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were then blocked with 5% non-fat milk in TBST [15 mM Tris-HCl (pH 7.4), 0.9% NaCl and 0.05% Tween-20 (pH 7.4)] for 1 h at room temperature, and then incubated with primary antibody overnight at 4°C using rabbit monoclonal anti-human BRCA1 antibody (dilution, 1:1,000; A301-377; Bethyl Laboratories, Montgomery, TX, USA), and goat anti-rabbit secondary antibody (dilution, 1:5,000; ZDR-5306; ZSGB-BIO, Beijing, China). Immunoreactive bands were detected using Super Signal West Femto Chemiluminescent Substrate (Merck KGaA, Darmstadt, Germany). The aforementioned experiments were performed at least three times with consistent results.

Cell proliferation assay. The cell proliferation assay was performed using cell counting kit-8 assay in total. 2x10⁵ cells were cultured in 96-well culture plates. The cells were resuspended in RPMI-1640 medium containing 10% FBS and cultured for 0, 24, 48 or 72 h at 37°C. The number of viable cells was determined by measuring absorbance at 450 nm using FLUOstar OPTIMA (BMG Labtech, Offenburg, Germany), according to the manufacturer’s instructions. Each experiment was performed in triplicate.

Laser confocal fluorescence microscopy for subcellular localization. The cells were fixed in 4% paraformaldehyde for 30 min at room temperature and lysed with 0.2% Triton-X-100 for 15 min. This was followed by the addition of 5% bovine serum albumin (ZLI-9027, ZSGB-BIO, Beijing, China) to the lysate and incubation for 30 min at 37°C. Rabbit monoclonal anti-human BRCA1 antibody (dilution, 1:1,000; A301-377; Bethyl Laboratories) and monoclonal antibody for histone H2A variant X (γH2AX; dilution, 1:100; 05-636-I; EMD Millipore, Billerica, MA, USA) were added to the mixture following incubation at 4°C overnight. Cell lysate with 0.01 mol/l phosphate-buffered saline instead of the primary antibody was used as a negative control. Fluorescence-labeled secondary antibodies (dilution, 1:4,000; ZF-0311, ZF-0316; ZSGB-BIO, Beijing, China) were added to the mixture, which was then incubated in the dark at room temperature for 30 min. The mixture was further incubated with DAPI solution at room temperature for 5 min and then mounted in Tris-buffered glycerol solution. The subcellular localization of the proteins

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was determined using confocal laser scanning microscopy at a magnification of x12,50.

Flow cytometry analysis of apoptosis. A total of 3x10^5 HCC1937 cells were seeded in 6-wells culture plates in RPMI-1640 medium with 10% FBS. After 12 h, cells were transfected with the aforementioned plasmids using Lipofectamine 2000. Subsequent to transfection, cells were collected at 24, 48 and 72 h and stained with fluorescein isothiocyanate-conjugated Annexin V (BioVision, Inc., Milpitas, CA, USA) at room temperature for 30 min, followed by staining with propidium iodide (PI) 1 min prior to analysis for apoptosis by FACScan (BD Biosciences, San Jose, CA, USA).

Cell cycle analysis. Cell cycle stage was determined by flow cytometry using a cell-cycle assay kit (Ab139418, Abcam, Cambridge, UK). Briefly, HCC1937 cells were harvested by centrifugation with 300 x g, 5 min at 4°C, washed with PBS and fixed with cold 75% ethanol at 4°C overnight. The fixed cells were then stained with PI and RNaseA (Ab139418; Abcam, Cambridge, UK), according to the manufacturer’s instruction. Following a 30-min incubation in the dark, fluorescence-activated cells were sorted in a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA). The cells were distinguished as being at the G0/G1, G2/M and S phases of the cell cycle based on the fluorescence intensity, and the distribution of the cell-cycle stage was analyzed using ModFit software (BD Biosciences).

Statistical analysis. Results were expressed as mean ± standard deviation. One-way analysis of variance was used for statistical comparison among groups. Multiple comparison between the groups was performed using Bonferroni method. P<0.05 was considered to indicate a statistically significant difference.

Results

Bioinformatics analyses showed that G1763V and L1786P abolish the tumor suppression function of BRCA1. Two novel BRCA1 missense mutations in the BRCT domain were identified from the cohort of Chinese women with familial breast cancer, consisting of G1763V due to p5407 G>T and L1786P due to p5476 T>C. Protein mutation analysis software, consisting of PolyPhen (http://genetics.bwh.harvard.edu/pph/), SIFT (http://sift.jcvi.org/) and Pmut (http://mmb2.pcb.ub.es:8080/PMut/), was used to predict the impact of these two BRCT mutations on BRCA1 protein and their possible pathogenicity. PolyPhen software predicted that G1763V and L1786P both damage the original protein function. SITF and Pmut software predicted that both mutations were deleterious (Table I).

G1763V and L1786P mutants were expressed in the BRCA1-deficient HCC1937 cell line. In order to test and verify the function of G1763V and L1786P mutations, mutation plasmids were constructed from pcDNA3.1-BRCA1 (termed wt BRCA1), and the expression of wild and mutant BRCA1 was examined in the BRCA1-deficiency breast cancer HCC1937 cell line. Fig. 1A shows the schematic diagram of the location of the mutants. The sequences of the mutant constructs were verified by sequencing (Fig. 1B). BRCA1 overexpression was observed both at the mRNA level and protein level in these three groups compared with the control group 24 h subsequent to transfection with wt BRCA1 and two mutants (Fig. 1C and D).

G1763V and L1786P mutants reduced cell growth and increased cell apoptosis in vitro. Subsequent to successfully constructing the transient transfection cell model, the effect of BRCA1 mutants on breast cancer cell growth and apoptosis was assessed in vitro. Cell proliferation assay using HCC1937 cells showed that the wild type BRCA1 and both of the two mutants significantly reduced the growth of breast cancer cells compared with the control group (P<0.05; Fig. 2A). Additionally, flow cytometric analysis showed that the wild type BRCA1 and both of the mutants significantly increased cell apoptosis compared with the control group (Fig. 2B and C) 72 h subsequent to transfection. These data indicated that G1763V and L1786P have similar tumor suppressor function to wild-type BRCA1 in vitro.

G1763V and L1786P mutants caused S-phase arrest after irradiation. In order to investigate the function of these two mutants following DNA damage, a DNA damage model was created using γ radiation. Phosphorylation of γH2AX is the most sensitive marker that can be used to examine the DNA damage and the subsequent DNA repair. It was found that γH2AX foci increased significantly in the irradiated group when compared with the groups without irradiation (Fig. 3A). After 8-Gy irradiation, the percentages of cells in the S-phase were 42.1, 42.1 and 40.0% in the wild-type BRCA1, G1763V and L1786P cells, respectively, which were increased compared with the control group with 29.2% (control vs. wt BRCA1, control vs. G1763V, control vs. L1786P; P<0.05; Fig. 3B).

G1763V and L1786P mutants did not sensitize cells to the PARP1 inhibitor Olaparib. In order to test the optimal concentration of Olaparib, HCC1937 cells were cultured in the presence of 0, 20, 40, 80 or 160 µM Olaparib. After 48 h, it was observed that 80 µM Olaparib produced the maximum suppression of PARP expression by 50% (Fig. 4A). Olaparib (80 µM) was then added into the cell cultures when the transfected cells were adherent. Olaparib increased cell apoptosis in HCC1937 (control 0 µM vs. control 80 µM; P<0.05). However, no synergistic effect between the Olaparib and BRCA1 mutation was noted on cell apoptosis (Fig. 4B).

Discussion

BRCA1 is one of the best-known tumor suppressor genes in breast cancer (8,13). Numerous mutations of BRCA1 have been reported (14-17), but the function of many of these mutations has not been studied. The present study identified two novel missense mutations of the BRCT domain from a cohort of Chinese Han patients with familial breast cancer, and explored the function and clinical significance of the mutations.

The key function of BRCA1 is to suppress tumor formation in breast and ovarian tissues. Using a mouse model of hereditary breast cancer, Shakya et al (7) found that the tumor
suppression function of BRCA1 is dependent on the ability of the BRCT domain to bind to its phospho-ligands. The BRCT motif of BRCA1 forms a phospho-recognition domain that preferentially binds to the phosphorylated isoforms of repair proteins, Abraxas/CCDC98, BACH1/FancJ and CtIP, and thus has a critical role in tumor suppression (18,19). Numerous tumor-associated BRCA1 alleles have frame shift/nonsense mutations that eliminate one or both BRCT motifs. According to the bioinformatics analyses, it was speculated that G1763V and L1786P may damage the tumor suppressor function of BRCA1. Notably, it was found that these two mutants had a similar tumor suppression function to wild-type BRCA1, in terms of reducing proliferation and inducing apoptosis in breast cancer cells.

PARP1 is a nuclear protein that rapidly binds to single-stranded DNA breaks to facilitate DNA repair (20). Inhibitors of PARP efficiently shrink breast, ovarian or prostate tumors in patients carrying hereditary mutations in the homologous recombination genes BRCA1 or BRCA2 (21-24). Using the BRCA1-deficient HCC1937 breast cancer cell line, the present results showed that G1763V and L1786P mutants were not sensitive to the inhibition of PARP inhibitor Olaparib. This data further indicated that these two mutations had no deleterious function.

These two mutants have no effect on the suppressor function of BRCA1, the reason for which requires additional investigation. Using the protein structure software, it was found that the G1763V and L1786P missense mutations were far away from the interaction site between BRCA1 and its phosphor-ligands (data not shown), which may at least partially explain why these two mutants have no deleterious effect on BRCA1 function.

In summary, the present study identified two novel BRCA1 missense mutations in the BRCT domain, G1763V and L1786P, and these two mutations did not affect the tumor suppressor function of BRCA1. It was concluded that not all BRCA1 missense mutations are pathogenic and that any new BRCA1 mutation should be assessed for its effect on the tumor suppressor function of BRCA1.

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Figure 2. Effect of G1763V and L1786P mutants on HCC1937 cells in cell growth and apoptosis. (A) Growth curve of HCC1937 cells with over expression of the two mutants. (B) Flow cytometry of HCC1937 in apoptosis at 72 h after transfection. The dot plots showed the percentage of apoptosis. (C) Bar graph showing apoptosis in the different groups studied. Bars indicate the mean ± standard deviation of triplicate results. *P<0.05. BRCA1, breast cancer 1; wt, wild type; OD, optical density; PI, propidium iodide.

Figure 3. Cell cycle of S-phase arrest by BRCA1 mutation after irradiation. (A) Subcellular localization of γH2AX after 8-Gy irradiation by immunofluorescence. (B) Cell-cycle stages were showed in the different groups studied. γH2AX, histone H2A variant X; BRCA1, breast cancer 1; wt, wild type.

Figure 4. No synergistic effect between Olaparib and BRCA1 mutations. (A) The mRNA expression of PARP in HCC1937 with 0, 10, 20, 40, 80 and 160 µM Olaparib. (B) Bar graph showing apoptosis in the different groups studied. Bars indicate the mean ± standard deviation of triplicate results. PARP, poly (ADP-ribose) polymerase 1; BRCA1, breast cancer 1; wt, wild type.
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