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ORIGINAL ARTICLE

Functional analysis of *BRCA1* missense variants of uncertain significance in Japanese breast cancer families

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Germline mutations in the tumor suppressor genes *BRCA1* and *BRCA2* are responsible for a large proportion of familial breast cancer cases, and therefore, *BRCA1* and *BRCA2* genetic testing has become increasingly common in clinical practice. However, variants of uncertain significance (VUS) have been detected in 16.3% of Japanese patients suspected of having hereditary breast and ovarian cancers. The clinical importance of VUS is unknown, and their incidence has led to issues in risk counseling, assessment and treatment of cancer patients. In the present study, we performed functional analysess of two VUS in *BRCA1*, A1752G and Y1853C that were detected in two independent breast cancer patients who were suspected of having hereditary breast cancer. Segregation analysis revealed that Y1853C, but not A1752G, was cosegregated in affected family members. Conservation, transcription and structure analyses also supported the pathogenic potential of Y1853C. Detailed segregation and *in silico* and *in vitro* analyses will enhance our understanding of VUS and improve the management of cancer patients and their families.
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INTRODUCTION

Breast cancer is the most common malignancy among Japanese women.[1] According to the Center for Cancer Control and Information Services (National Cancer Center, Japan), breast cancer affects 1 in 16 Japanese women. Breast cancer is the fifth most common cause of cancer-related deaths in Japan, accounting for 11 177 female deaths in 2006.[1] Both genetic and environmental factors contribute to the development of breast cancer. Familial aggregation increases breast cancer risk two to four-fold by the age of 70 years.[2] Germline mutations in *BRCA1* and *BRCA2* have been linked to hereditary breast cancer.[3,4] *BRCA1* and *BRCA2* are involved in DNA repair, gene transcription and the cell cycle.[5] *BRCA1* encodes a 220-kDa protein that has a RING finger N-terminal domain and a BRCA1 C-terminal (BRCT) domain.[5] The BRCT domain is a founding member of the large family of BRCT motif-containing proteins that are known to be involved in DNA repair.[6] BRCA2 has BRC repeats and interacts with RAD51, which is involved in DNA homologous recombination and double-strand break repair.[7–9] *BRCA1* and *BRCA2* have important roles in tumor suppression.[7] Approximately, 45–85% of subjects with *BRCA1* and *BRCA2* germline mutations develop breast and/or ovarian cancer by the age of 70 years. Furthermore, *BRCA1* and *BRCA2* mutation carriers diagnosed with breast cancer have an increased risk of developing contralateral second primary breast cancer and ovarian cancer.[2] Therefore, it is useful to evaluate *BRCA1* and *BRCA2* germline mutations to determine treatment decisions for patients and genetic counseling for at-risk relatives. There are several databases for *BRCA* variants such as the Breast Cancer Information Core (http://research.nhgri.nih.gov/projects/bic/Member/index.shtml), Leiden Open Source Variant (http://chromium.liacs.nl/LOVD2/cancer/home.php), Evidence-based Network for the Interpretation of Germline Mutant Alleles (http://www.enigmaconsortium.org) and the Universal Mutation Database (UMD; http://www.umd.be/BRCA1/). According to the UMD, 5821 mutations in *BRCA1* and *BRCA2* have been reported. Variants of uncertain significance (VUS) have been detected in 16.3% of Japanese women undergoing *BRCA1* and *BRCA2* genetic testing.[10] Most of these VUS mutations are in-frame deletion or insertions, missense or silent mutations, or alterations in intronic and regulatory regions. The clinical significance of VUS is uncertain; therefore, classification of VUS mutations as either pathogenic or benign is important. In the present study, we examined the *BRCA1* and *BRCA2* germline mutations in five Japanese women with breast cancer. We identified two VUS mutations of *BRCA1* (A1752G and Y1853C) in two patients, and performed a functional analysis of

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these VUS mutations for the clinical assessment of patients and their at-risk family members.

MATERIALS AND METHODS

Subjects

Five Japanese women with breast cancer were subjected to *BRCA1* and *BRCA2* full-sequence analysis. The patients were assigned to the matrix chart based on the reported prevalence of *BRCA1* and *BRCA2* mutations in 3011 non-Ashkenazi individuals in the USA[11] (Supplementary Table) and classified into Groups I-1 to IV-3 (Table 1). Two breast cancer families with VUS were enrolled for further analysis (Figure 1). In family 1, a 46-year-old woman was diagnosed with primary breast cancer. Her father and sister were also affected by breast cancer. Family 2 had three members with breast cancer and two members with ovarian cancer. All exons and exon–intron boundaries of *BRCA1* and *BRCA2* in each proband were sequenced at FALCO Biosystems (Kyoto, Japan). Additional sequencing of *BRCA1* mutations in the patients' family members was also performed by FALCO Biosystems. This study was approved by the Ethical Committee of the University of Tsukuba, and written informed consent was obtained from each participant.

Table 1 The probands classified as the groups

Family	*Individuals*	*Mutation*	*Exon*	*dbSNP ID*	*Groups*[a]	*Subgroups*[a]
1	II-2	A1752G	20	NR	II	II-2
2	IV-1	Y1853C	24	rs80357258	IV	IV-3
3	III-4	ND	—	—	II	II-2
4	II-2	ND	—	—	I	I-1
5	III-1	ND	—	—	I	I-1

Abbreviations: ND, not detected; NR, not reported.
[a]The groups and subgroups are from the study by Sugano *et al*.[10]

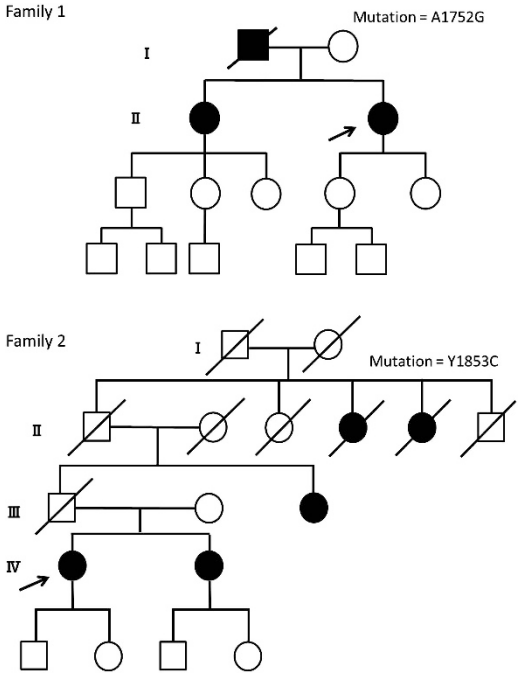

Figure 1 Pedigrees of breast cancer families with *BRCA1* VUS. The arrow indicates the proband. Closed circles or squares indicate affected individuals.

Transactivation assay

For the functional analysis of *BRCA1* VUS, transactivation assay was carried out using the pGL4.31 vector (Promega, Madison, WI, USA), which contains a firefly luciferase gene under the control of GAL4-binding sites; the pBIND vector (Promega), which contains amino acids 1396–1863 of human BRCA1 (U14680);[12] a GAL4 DNA-binding domain and a Renilla luciferase gene. M1775R, A1752G and Y1853C mutations were introduced into pBIND with the BRCA1–GAL4 fusion protein using the PrimeSTAR Mutagenesis Basal Kit (Takara, Shiga, Japan). A BRCT deleterious variant, M1775R, was used as a positive control.[12] 293T cells (Riken Bioresource Center, Ibaraki, Japan) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and plated in 24-well plates the day before transfection. pGL4.31 and pBIND constructs were transfected into 293T cells using Lipofectamine (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Transfections were carried out in triplicate. Transfections were normalized with an internal control of pBIND, which contains a Renilla luciferase gene, using the Dual-Luciferase Reporter Assay System (Promega). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to assess the GAL4–BRCA1 complex. Cells were harvested 24 h after transfection and lysed. Equal amounts of cell lysate protein were loaded onto a 10% SDS-PAGE gel and electroblotted onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) using a semi-dry apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Immunoblotting was performed using a GAL4 DNA-binding domain antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) with an anti-mouse HRP-conjugated secondary antibody (1:10000 dilution; Medical & Biological Laboratories, Aichi, Japan). A monoclonal β-actin antibody was used as an internal control (Sigma-Aldrich, St Louis, MO, USA). Images were acquired using the Luminescent Image Analyzer LAS-4000 mini (Fujifilm, Tokyo, Japan). The Multi Gauge software (Fujifilm) was used to quantify the intensity of the band on a western blot. Correlation of the transcription activation levels with GAL4–BRCA1 protein levels was evaluated using the Pearson correlation coefficient.

BrdU proliferation assay

Cell proliferation assay was performed using the BrdU Cell Proliferation ELISA Kit, BrdU (Roche, Basel, Switzerland) according to the manufacturer's instructions. Briefly, 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 1×10^4 cells per well and plated on 96-well plates 1 day before transfection. pBIND constructs were transfected into 293T cells as described above and incubated for an additional 20 h. BrdU (100 μM) was added to each well and incubated for 24 h. Cells were fixed using FixDenat, and anti-BrdU-POD working solution was added to each well and incubated for 90 min. The plates were washed with the washing solution, and the substrate solution was added to each well. The reaction was stopped by adding 2 M sulfuric acid, and absorbance was then measured at 450 nm.

In silico analysis of amino acid substitutions

Sorting Intolerant from Tolerant (SIFT) software[13] (http://sift.bii.a-star.edu.sg/) was used to predict whether an amino-acid substitution affected protein function based on sequence homology and the physical properties of amino acids. Amino-acid substitutions were classified as intolerant (0.00–0.05), potentially intolerant (0.051–0.10), borderline (0.101–0.20) or tolerant (0.201–1.00). Polymorphism Phenotyping version 2 software (PolyPhen-2)[14] (http://genetics.bwh.harvard.edu/pph2/) was also used to predict the potential effect of amino-acid substitutions on protein structure and function based on sequence, evolutionary conservation and structural information.

Structure analysis

Modeller[15] (http://www.salilab.org/modeller/) was used to model the BRCA1 mutant structures. The template BRCA1 structure file was from PDBj (ID: 1t29, Protein Data Bank Japan), and PyMol (www.pymol.org) was used as the graphic software. The wild-type side chain was computationally replaced with A1752G or Y1853C, and the conformation of the variant backbone and side chain atoms were optimized based on the hydrophobicity analysis of the

protein surface, the distance between amino acids and the amino-acid characteristics.

RESULTS

Mutation screening of the proband in *BRCA1* and *BRCA2* identified two VUS in the BRCT domains of *BRCA1* (A1752G in II-2 of family 1 and Y1853C in IV-1 of family 2; Figure 1). The A1752G variant has not been reported previously, whereas the Y1853C variant has been deposited at dbSNP (rs80357258), although the frequency of this mutation is unknown. In family 1, A1752G was not detected in her sister suffering from breast cancer (II-1), and the DNA of another affected family member (I-1) was not available. In contrast, in family 2, the Y1853C mutation was detected in III-3 and IV-2 and cosegregated among the affected family members.

In silico analysis revealed that A1752G and Y1835C had SIFT scores of 0.02 (intolerant) and 0.00 (intolerant), respectively, and PolyPhen-2 scores of 0.172 (benign) and 1.0 (probably damaging), respectively (Supplementary Figure 1). As these two variants were located in the BRCT domain, transactivation assays were performed to examine their functional effects. A1752G and Y1853C reduced transcription activation levels in human 293T cells (A1752G, $P = 7 \times 10^{-6}$ and Y1853C, $P = 2 \times 10^{-4}$; Figure 2). The expression levels of GAL4–BRCA1 complexes were correlated with the levels of transcription activation ($R^2 = 0.95$, $P = 0.012$). The cell proliferation assay using plasmid constructs with the *BRCA1* mutation showed no differences in cell proliferation between the wild-type allele and the mutated allele ($P > 0.05$; Supplementary Figure 2).

Structure analysis using Modeller is shown in Supplementary Figure 3. A1752 was located on the α-helix in the BRCT domain and not on the protein surface or surface cleft that interacts with phosphorylated ligands.[16] Moreover, significant structural changes were not expected, because alanine and glycine have similar chemical properties. Y1853 was also located on the α−helix in the BRCT domain. This position is not critical to the surface cleft;[16] however, the cysteine substitution has the potential to form a disulfide bond because the expected distance between 1847C and 1853C is 9.8 Å (Supplementary Figure 3B).

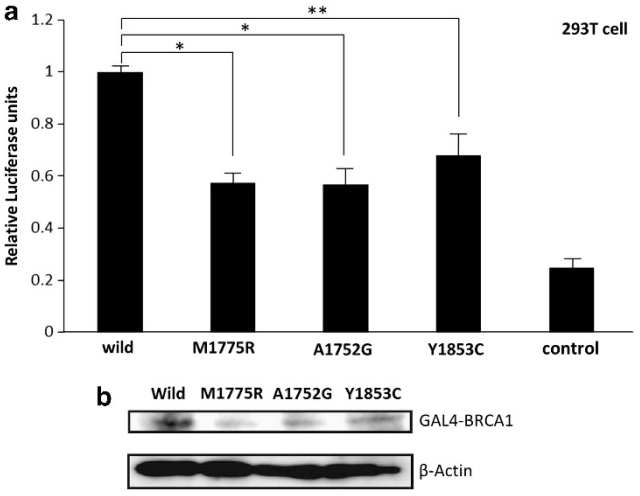

Figure 2 Transactivation assay of *BRCA1* VUS in human 293T cells. (**a**) Luciferase activity of wild-type BRCA1, M1775R (positive control), A1752G, Y1853C and empty vector in pBIND (negative control). The scores are plotted as fold activation compared with wild-type BRCA1. $^{*}P < 1 \times 10^{-5}$ and $^{**}P < 1 \times 10^{-3}$. (**b**) Immunoblot of GAL4-BRCA1 expression in human 293T using a GAL4 DNA-binding domain antibody. β-actin was used as the internal control.

DISCUSSION

Counseling of families in which only VUS mutations are detected is problematic because genetic testing results cannot be used to unambiguously identify at-risk family members, and the clinical management of breast cancer patients with VUS needs to be highly individualized.[11] In the present study, we performed a functional analysis of *BRCA1* VUS in two Japanese families to provide a more informative risk assessment for patients and their family members. A1752G has not been reported previously, but two mutations at the same position, A1752V and A1752P, were reported to be high-risk mutations.[12,17,18] SIFT score and transactivation assay results support the potentially damaging effect of A1752G. However, protein structure analysis by Modeller and *in silico* analysis by PolyPhen-2 revealed that A1752G was not likely to have a significant functional effect on BRCA1. Furthermore, this variant was not cosegregated in affected family members. Together, our findings suggest that A1752G is less likely to be a pathogenic mutation. In contrast, all functional analyses including transactivation assay, *in silico* assay and structure modeling indicated that Y1853C variants were likely to be deleterious mutations. Previous studies have also predicted that Y1853C is pathogenic, on the basis of *in silico* analysis,[19] transactivation assay[18] and proteolytic degradation assay.[20] The cysteine substitution has the potential to form a disulfide bond, and this may lead to instability of the BRCA1 structure, which may contribute to a decrease in BRCA1 expression.

In the present study, similar cell proliferation levels were observed using BRCA1 mutation constructs, in addition to the correlation between the reduced transcription activation levels and low expression level of the GAL4–BRCA1 complex. A previous study using yeast expression systems showed that fusion proteins containing human BRCA1, which includes the *M1775R* mutation, inhibit the growth of yeast, whereas western blot analysis demonstrated similar levels of expression for all constructs,[21] contradicting the results of the present study. A previous study by Phelan *et al.*[12] showed that protein expression levels in yeast systems and those in mammalian systems disagreed in some constructs (for example, V1809F and W1837R), although they had not conducted the experiment using the M1775R construct. Moreover, Carvalho *et al.*[22] performed a transcription assay for BRCA1 VUS variants both in yeast and mammalian cells, and showed that the expression levels of several variants, including M1775R, decreased in mammalian cells but not in yeast cells. Therefore, reduced transcriptional transactivation observed in the present study probably reflects the instability of mutant proteins in mammalian cells rather than affecting transactivation activities.

Integrated studies that include detailed segregation and *in silico* and *in vitro* analyses will improve our understanding of the role of VUS in cancer risk and in the management of cancer patients and their families.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)