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BRIEF REPORT

A recombination-based method to characterize human BRCA1 missense variants

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Abstract Many missense variants in BRCA1 are of unclear clinical significance. Functional and genetic approaches have been proposed for elucidating the clinical significance of such variants. The purpose of this study was to evaluate BRCA1 missense variants for their effect on both homologous recombination (HR) and non homologous end joining (NHEJ). HR frequency evaluation: HeLaG1 cells, containing a stably integrated plasmid that allows us to measure HR events by gene conversion events, were transfected with the pcDNA3β expression vector containing the BRCA1-wild-type (*BRCA1 wild type*) or the BRCA1-unclassified variants (BRCA1-UCVs). The NHEJ was measured by a random plasmid integration assay. The assays suggested a BRCA1 involvement mainly in the NHEJ. As a matter of fact, the Y179C and the A1789T variant significantly altered the NHEJ activity as compared to the wild type, suggesting that they may be related to BRCA1-associated pathogenicity by affecting this function. The variants N550H and I1766S, and the mutation M1775R did not alter the NHEJ frequency. These data, besides proposing a method for the study of BRCA1 variants' effect on HR and NHEJ, highlighted the need for a range of functional assays to be performed to identify variants with altered function.

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Introduction

Breast cancer is the most common neoplasia in women, and the second cause of death after cardiovascular diseases in the Western world. About 10% of breast cancer cases is inheritable, and about 40% of those is caused by mutations in *BRCA1* or *BRCA2* genes.

BRCA1 is a tumor suppressor gene, which encodes a nuclear protein involved in several cellular processes including DNA double strand break repair by homologous recombination (HR) and non homologous end joining (NHEJ), cell cycle control, apoptosis, and maintenance of the genomic stability [1–3]. *BRCA1* gene is highly polymorphic. Nonsense or frameshift *BRCA1* mutations encoding truncated, but not functional proteins, predispose women to early-onset breast and ovarian cancer. However, several missense variants of uncertain pathological significance have been identified.

A variety of predictive approaches have been reported to distinguish cancer-related variants from neutral polymorphisms. These methods are based on the degree of conservation among species, the nature and position of amino acid substitution, the analysis of co-segregation pattern of the variant with disease in affected family members, the inactivation of the wild-type allele either by loss of heterozygosity or by promoter hypermethylation in the tumor [4–6]. Moreover, several functional assays biologically evaluating the variant effect on the ability of the protein to perform some of the key cellular functions are currently used. They can potentially be used to predict whether the variant predisposes to disease or alternatively has no significant influence on cancer risk [7].

In this study, we used two functional assays in HeLa cells, which specifically evaluate the effect of the over-expression of the wild-type or mutated *BRCA1* on spontaneous HR and on random chromosomal integration of a linearized plasmid DNA, a subtype of non HR, to better elucidate the clinical relevance of some BRCA1 unclassified variants.

There are several evidences of BRCA1 involvement in DNA double strand break repair by HR. BRCA1 colocalizes with RAD51 protein into sub-nuclear complexes in mitotic cells, and clinical mutations at the C-terminal BRCA1 BRCT domain disrupt the nuclear foci localization [2]. Moreover, BRCA1-deficient cells are highly sensitive to ionizing radiation and display chromosome instability [8]. BRCA1$^{-/-}$ mouse embryonic stem cells have impaired HR [9]. On the other hand, even though BRCA1 binds in vitro and in vivo to Mre11//Rad50/Nbs1 complex [10], its role in NHEJ pathway has not yet been completely clarified. As a matter of fact, the frequency of random plasmid integration in transiently *BRCA1 wild type* transfected HCC1937 cells is significantly increased as compared to the parental cell line [11], whereas this phenomenon is also impaired in *BRCA1*$^{-/-}$ mouse embryonic fibroblasts, but contradictory results were obtained [10, 12, 13].

In this study, we selected some missense variants from a mutational screening of 276 breast and/or ovarian cancer families. Four non-synonymous variants, which localized in different BRCA1 functional domains, were identified as potentially deleterious and likely disrupting the gene function using three predictive software: SIFT, Polyphen, and Align-GVGD. These variants were the Y179C, the N550H, the I1766S, and the A1789T. One known missense variant (M1775R), previously reported as deleterious mutation, was chosen as positive control. We evaluated the effect of the over-expression of the wild-type or these mutated BRCA1 proteins on spontaneous HR and NHEJ events in HeLa cells.

Materials and methods

Samples and mutation selection

The DNA samples from 276 individuals, belonging to 276 breast and/or ovarian cancer families, collected at the University Hospital of Pisa, were analyzed for *BRCA1* and *BRCA2* germline mutations using an automated DNA sequencer (ABI 3100; Applera-Applied Biosystems). We used the following selection criteria:

(1) occurrence of two or more cases of breast and/or ovarian cancer in first or second degree relatives;
(2) early onset of the disease;
(3) occurrence of bilateral breast cancer or occurrence of breast and ovarian cancer in the same individual.

The screening revealed several known as well as novel unclassified variants (UCVs) localized across all the *BRCA1* gene sequence. To identify non-synonymous amino acid changes likely to disrupt BRCA1 gene function, three comparative evolutionary bioinformatic programs were used: sorting intolerant from tolerant (SIFT) [14]. http://blocks.fhcrc.org/sift/SIFT.html), polymorphism phenotyping (PolyPhen) [15]; http://tux.embl-heidelberg.de/ramensky/pilyphen.cgi and Align-GVGD (http://agvgd.iarc.fr/alignments) [16].

Plasmids

To determine whether the expression of *BRCA1*-wild-type or mutated, affects homologous and non-homologous recombination in human cells, we used pcDNA3–BRCA1 expression plasmid (a gift from David Livingston, Boston MA, USA) [2]. In this vector, the β-globin gene was inserted to optimize the expression of BRCA1 [2]. To express the BRCA1 missense variant Y179C, N550H, A1789T, and I1766S and the pathogenic control M1775R, we constructed the corresponding pcDNA3–BRCA1 derivative vector by site-specific mutagenesis using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene Inc) following the protocol recommended by the manufacturer. To measure the effect of the expression of *BRCA1 wild type* or mutated on random plasmid integration, we used the plasmid pBlue-puro (a kind gift from Roland Kanaar, Erasmus University, Rotterdam, NL) that contains the puromycin resistance gene driven by cytomegalovirus promoter.

Cell culture and transfection

HeLaG1 and HeLa cell lines were routinely cultured in Dulbecco's modified Eagle's medium, DMEM (GIBCO), supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, and 100 mg/ml streptomycin (GIBCO). Cultures were incubated at 37°C in 5% CO_2 and 95% relative humidity. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The efficiency of transfection was determined using the pGFP plasmid (a gift from Giuseppe Rainaldi, Pisa Italy) followed by direct count of GFP positive cells by FACS analysis (Becton–Dickinson Biosciences). Usually, the efficiency of transfection ranged from 70 to 85%.

Immunoblotting

Twenty-four hours after transfection of pcDNA3BRCA1, aliquots of 4 × 10^5 cells were washed twice in phosphate buffered saline (PBS) 1× and lysed in the Laemmli sample buffer 1× (Tris–HCl 50 Mm pH 6.8, SDS 2%, glycerol 10%, bromophenol blue 0.1%, β-mercaptoethanol 100 mM) together with the Protease Inhibitor Cocktail 1× (Sigma). The protein extracts were denaturated at 100°C for 5 min. A total of ~120 μg of whole cell extract was subjected to electrophoresis at 10–20 mA for ~3 h in 6% SDS-polyacrylamide gel; thereafter, the proteins were transferred to polyvinylidene fluoride membrane at 170 mA for 17 h at 4°C using a Mini-PROTEAN® Cell apparatus (Bio-Rad). BRCA1 was detected using anti-BRCA1 monoclonal antibody Ab4 (Calbiochem, Gibbstown, NJ) diluted 1:100 with 3% of BSA. This antibody recognizes aa 1005–1313 in the exon 11 of the BRCA1 protein. Anti-mouse horseradish peroxidase-linked antibody (Amersham Biosciences, Piscataway, NJ), diluted 1:15,000, was used as a secondary antibody. The BRCA1 protein was detected using the ECL chemioluminescence solution (Bio-Rad), and the signals were developed on photographic films (Sigma).

Homologous recombination assay

The HeLaG1 cells (a gift from Margherita Bignami, Rome Italy) contain a stably integrated plasmid that allows to measure gene conversion events between two differentially mutated hygromycin-resistance (Hyg^R) genes [17]. One Hyg^R gene is mutated at the *Pvu*I site (*hyg1*), and the other Hyg^R at the *Sac*II site (*hyg2*) (Fig. 3). An intrachromosomal recombination event leads the restoration of wt Hyg^R gene; therefore, the frequency of intrachromosomal recombination was calculated as total number of Hyg^R clones × 10^{-5} viable cells. HeLaG1 cells were transfected with the pcDNA3β expression vector containing the wild-type *BRCA1* or the *BRCA1*–UCVs. 24 h after transfection, cells were harvested and plated [6 × 10^5 cells/10-cm dish and 10^2 cells/6-cm dish, for plating efficiency (PE) evaluation]. For the selection of the recombination events, 24 h later, we added hygromicin 0.2 mg/ml (Sigma) to the medium. Medium was changed twice and, after 10–15 days, plates were stained with crystal violet, and clones were counted [18].

Random plasmid integration assay

The effect of BRCA1 expression on NHEJ was determined, as previously reported, by testing the effect of these proteins on random plasmid integration in HeLa cells [19]. The frequency of NHEJ was determined by co-transfecting the HeLa cells with 2 μg of the pcDNA3β. *BRCA1 wild type* or *BRCA1-UCV* vectors and 2 μg of pBlue-puro that carries no homology with the genome of HeLa cells so that it stably integrates by non-homologous recombination [19]. One day after transfection, cells were collected and plated (2 × 10^5 cells/dish) in 10-cm dishes containing 0.2 μg/ml puromycin. Culture medium was changed after 7 days and replaced with puromycin-free fresh medium. The colonies were stained and counted 7 days later, and the frequency of recombination was calculated by dividing the number of puromycin-resistant colonies by the number of seeded cells corrected by the plating efficiency.

Statistical analysis

The frequency of Hyg^R clones obtained after the transfection of the empty-vector was used as reference. The results were analyzed by the *t*-Student test. All the analyses were performed using Statgraphics (StatPoint Inc. USA).

Results

Variants selection

We selected four non-synonymous UCVs, suggested by SIFT, Polyphen, and Align-GVGD software, as likely capable of disrupting the protein function: the Y179C, the N550H, the I1766S, and the A1789T (Table 1, Fig. 1) identified in 4 out of 276 breast and breast-ovarian cancer families.

The M1775R, classified as deleterious, was used as positive control [20]. The A1789T variant has never been described previously. It was found in one family. The proband was affected by breast cancer at 32 years of age. The mother of the proband, affected by breast and ovarian cancer diagnosed at 46 and 50 years of age, respectively, was found to be a carrier of the variant (Fig. 2a). The I1766S was classified as a deleterious amino acidic change by Carvalho et al. [21]. It was found in one family, and the proband had ovarian carcinoma diagnosed at 42 years of age. A DNA sample was available from a sister of the proband unaffected at 50 years of age. She tested negative for the mutation (Fig. 2b).

The Y179C was classified as neutral by Judkins [22]. The N550H was classified as probably neutral by Tavtigian [16]. These two UCVs were inherited together with the polymorphism F486L in two apparently unrelated families (Fig. 2c). The proband from one family was affected by breast cancer at 42 years of age. Two-second-degree relatives in the paternal branch, the proband's grandmother and a cousin, were affected by breast cancer. The affected cousin was found negative for the variants. The proband from the other family was affected by bilateral metacronous

Table 1 Description of the BRCA*2* missense variants analyzed

[a]BRCA1-UCV	[b]BRCA1-UCV HGVS	[c]Families harboring the UCV	[d]BIC recorded (no. of times)
Y179C	p.Tyr179Cys	2	54
N550H	p.Asn550His	2	54
I1766S	p.Ile1766Ser	1	7
A1789T	p.Ala1789Thr	1	0

[a] The UCV nomenclature as in BIC database (http://research.nhgri.nih.gov/bic)

[b] The UCV nomenclature following the journal guidelines (www.hgvs.org/mutnomen)

[c] The number of the families harboring the UCV

[d] Number of probands recorded in the BIC database

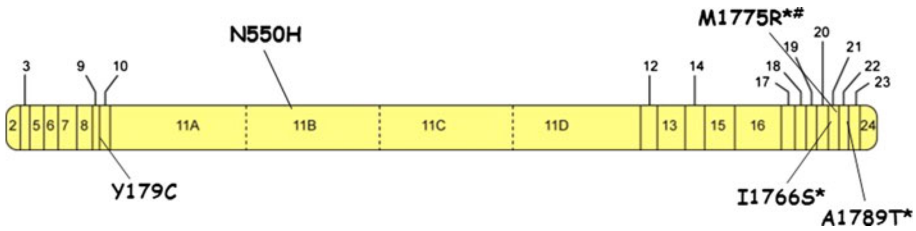

Fig. 1 Localization of the UCVs in the BRCA1 cDNA sequence. The *numbers* indicate the exons. * Mutation localized in BRCT domain, # pathogenetic control variant

breast cancer at 48 and 53 years of age. The proband's mother and two cousins were affected by breast cancer. Unfortunately none of them was available for mutation testing.

Functional assays

Homologous recombination in HeLa cells

In order to set up a novel functional assay to distinguish between neutral polymorphisms and deleterious mutations, we created several vectors derived from pcDNA3*β-BRCA1 wild type*, by site-directed mutagenesis, each of them expressing a selected UCV. These vectors were transfected in the HeLaG1 cells that carry a recombination substrate measuring intrachromosomal recombination events between the mutated *hyg1* and *hyg2* alleles (see "Materials and methods," Fig. 3). First, we checked whether the expression of the wild-type and mutated *BRCA1* was detectable 24 h after transfection. Then, we prepared the total lysate, as described in the methods, and carried out Western blot analysis. The Fig. 4 shows that all the proteins were expressed roughly at similar level as compared to the α-tubulin, suggesting that the proteins are equally stable in the cells. Importantly, the transgene expression was clearly detectable in the blot after few minutes of exposure, when the endogenous BRCA1 was not visible (Fig. 4). The expression of endogenous BRCA1 was seen only after 2 h of exposure (data not shown). Thus, under these conditions, we concluded that the exogenous BRCA1 proteins were over-expressed. This prompted us to determine whether this transient expression of the BRCA1 protein affected recombination. For this reason, 24 h after transfection, the cells were seeded in the presence of hygromicin to score for intrachromosomal recombinants. Under these conditions, the wild type increased the recombination frequency of 1.6-fold compared to the empty vector, and this difference was statistically significant (*t*-test $P < 0.005$) (Table 2): the HR frequency of HeLa G1 cells transfected with empty vector was $5.99 \pm 2.3 \times 10^{-5}$ viable cells. All the UCVs tested showed an increase in HR ranging from 0.98- to 1.3-fold compared to the empty vector. Thus, a functional assay based on HR in human cells would not presumably be helpful to characterize BRCA1 UCVs.

Random plasmid integration in HeLa cells

To evaluate whether BRCA1 UCVs had an influence on NHEJ, we determined the effects of the expressions of these proteins on random (non-homologous) plasmid integration in HeLa cells. The plasmid expressing the *BRCA1 wild type* or *BRCA1* UCVs was co-transfected with the pBlue-puro plasmid; after 24 h, the puromycin was added, and the frequency of random plasmid integration was measured as number of puromycin-resistant clones on 10^3 viable cells.

The expression of exogenous wild-type and the mutant I1766S BRCA1 protein increased the plasmid random integration in HeLa by 2.3- and 2.5-fold respectively as compared to the control (Table 2). The over-expressions of the mutant BRCA1 protein N550H and the M1775R stimulated the plasmid random integration by 3.1- and 3.2-fold, respectively, as compared to the control (Table 2).

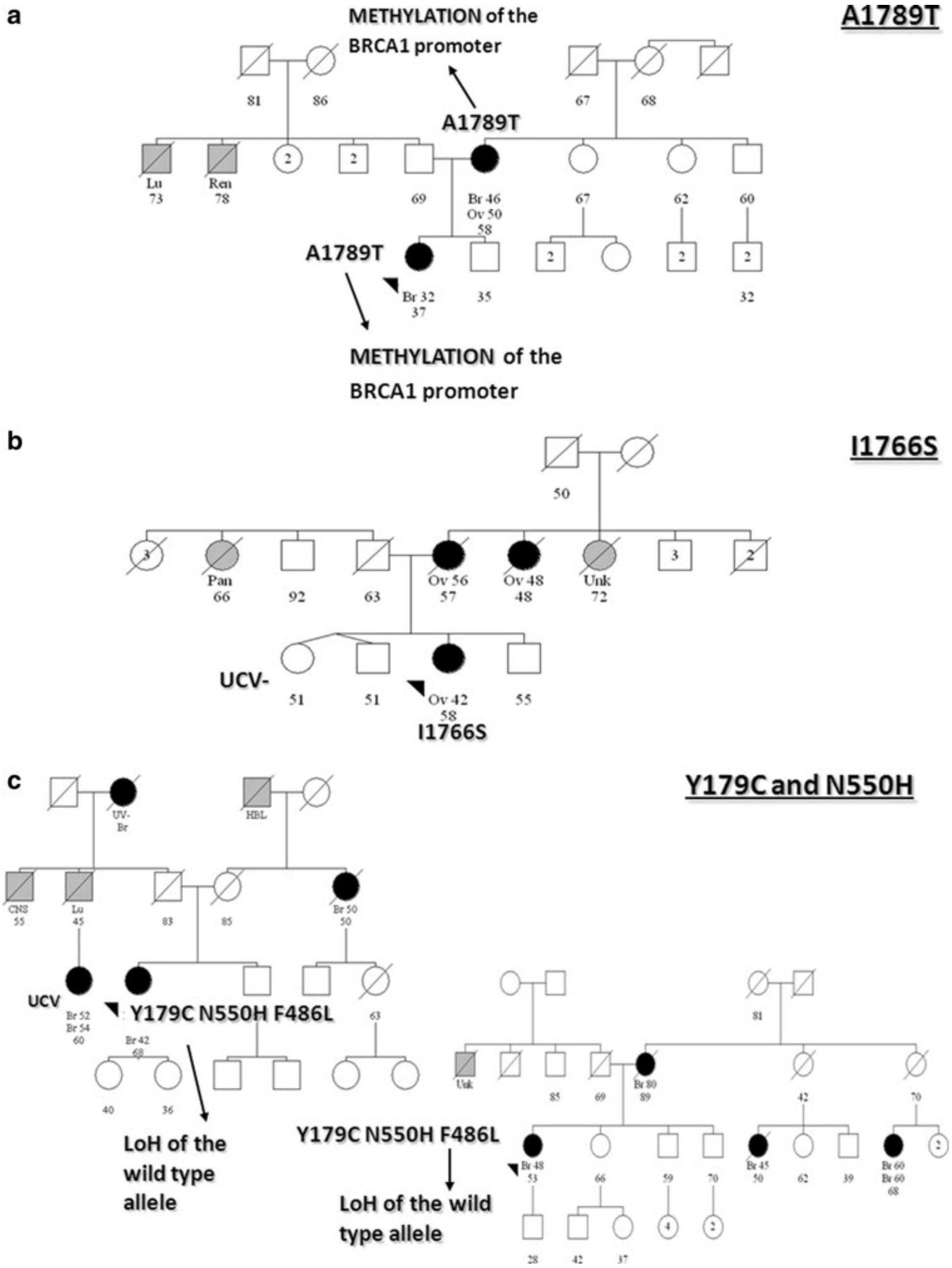

Fig. 2 Pedigrees of families harboring the variants: A1789T (**a**), I1766S (**b**), and Y179C and N550H (**c**)

The over-expression of variants Y179C and A1789T induced the highest increase of plasmid random integration by 3.5- and 4.6-fold respectively as compared to the control ($P \leq 0.001$). In conclusion, the I1766S and the M1775R UCVs behaved similarly to the *BRCA1 wild type*, whereas the Y179C and the A1789T induced a significant increase of random integration (Table 3).

Discussion

Only a very small fraction of *BRCA1* missense variants have been classified either as deleterious or neutral, while the majority remain as unclassified variants significance (UCVs). Interpreting such variants poses significant challenges for both clinicians and patients. To predict the clinical relevance of unclassified variants, several approaches are recommended. Bioinformatic prediction software supported by functional assays, classical genetic analysis and tumor phenotype, are useful to produce a prediction algorithm as proposed by Golgdar and Tavtigian [23, 24]. However, in general, it is easier to conclude that a variant is non-pathogenic than pathogenic [25].

BRCA1 acts as a tumor suppressor gene, and germ-line mutations which disrupt its functions culminate, after the loss of the wild-type allele, in cancer development.

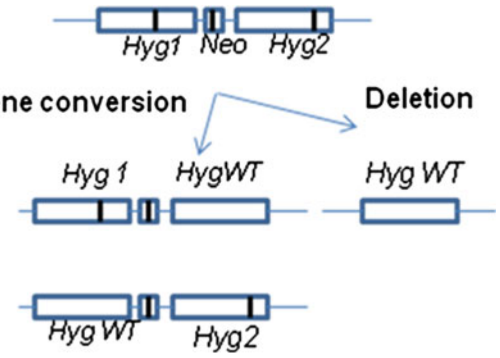

Fig. 3 The intrachromosomal recombination in human cells. HeLaG1 cells contain two copies of HygR genes inactivated by 10-bp insertions, either at a unique *Pvu*I site (*hyg1*) or at a unique *Sac*II site (*hyg2*); the two mutated *hyg* genes are in direct repeat orientation and are separated by a sequence containing the amino-glycoside phosphotransferase (Neo) gene conferring resistance to G418; an intrachromosomal recombination event occurring by gene conversion between the two *hyg* sequences results in restoration of one of the mutant *hyg* genes to wild type; the intrachromosomal deletion of the DNA sequence between the two mutated *hyg* genes leads to the formation of a HygR wild type (Hyg WT) with loss of intervening sequence; the intrachromosomal recombination was measured after transfecting HeLaG1 cells with either *BRCA1 wild type* or UCVs

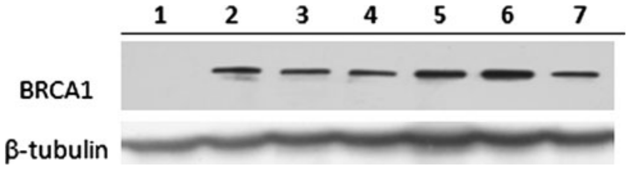

Fig. 4 Western blot analysis to measure the expression of the *BRCA1 wild type* and UCVs protein in a HeLa cell line extract. The monoclonal Ab-4 antibody specifically directed towards exon 11 BRCA1 protein was used, as well as the polyclonal anti-*β*-tubulin control antibody. *1* pcDNA3.1, *2* pcDNA3–BRCA1 wt, *3* pcDNA3–BRCA1-M1775R, *4* pcDNA3–BRCA-A1789T, *5* pcDNA3–BRCA1-I1766S, *6* pcDNA3–BRCA1Y179C, and *7* pcDNA3–BRCA1-N550H

Table 2 Effect of the expression of wild-type or mutated BRCA1 on spontaneous intrachromosomal recombination and random plasmid integration in HeLa cells

Protein expressed	[a]Intrachromosomal recombination Hyg^R clone/10^5 viable cells	[b]Random integration Puro^R clones/10^3 viable cells
Control	5.99 ± 2.3 (96) [1]	0.9 ± 0.3 (265) [1]
Wild type	9.1 ± 3.8 (159) [1.6]*	2.1 ± 0.8 (533)[2.3]**
Y179C	6.53 ± 2.3 (78) [1.1]	3.2 ± 1.1 (749) [3.5]***
N550H	7.4 ± 3.2 (90) [1.3]	2.8 ± 1.5 (697)[3.1]*
I1766S	5.80 ± 2.66 (63) [1.03]	2.3 ± 1.2 (625)[2.5]*
M1775R	5.7 ± 1.35 (105) [0.98]	3.0 ± 1.5 (648)[3.2]**
A1789T	7.97 ± 3.7 (150) [1.3]	4.2 ± 1.4 (789)[4.6]***

[a] Intrachromosomal recombination was determined in HeLa G1 cells after transient expression of BRCA1

[b] The random integration was assessed in HeLa cells after co-transfection of the BRCA1 plasmid and the pBlu-puro plasmid

The results are reported as mean of six experiments ± standard deviation. The numbers in the *round brackets* represent the total number of the counted clones. In the *square brackets*, we show the fold increase over the control. The control is the empty vector. Results are statistically analyzed with the Student *t*-test

$*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$

Table 3 Comparison of effects *BRCA1 wild type* and UCVs on several recombination-based functional assays

Mutated protein	HR in yeast	HR in HeLa	RI in HeLa
Y179C	+	−	+
N550H	−	−	−
I1766S	+	−	−
M1775R	+	−	−
A1789T	−	−	+

The results with reference to homologous recombination in yeast are reported in Caligo et al. [30]. *RI* random integration. The variant is scored as + when the results obtained are statistically different from the wt, and as − when the results are not different from the wt

Although its precise biochemical functions, relevant for tumor suppression, still remains to be clarified, BRCA1 has been demonstrated to play a role in several cellular processes including DNA double strand breaks repair, transcriptional regulation, chromatin remodelling, cell–cycle checkpoint control, protein ubiquitination, and centrosome replication [26].

Several functional assays have been used to distinguish between BRCA1 cancer-related mutations and neutral polymorphisms, but due to its multitasking characteristic, there is no comprehensive functional assay available for BRCA1 [6, 27]. In this article, we propose two functional assays: the first one based on transient expression of the UCVs in HeLa G1 cells containing a HR substrate, and the second one on random chromosomal integration of a linearized plasmid DNA in the genome of HeLa cells transiently expressing the UCVs. We studied a total of five BRCA1 missense variants of which one was already classified as pathogenic and used as a control. The variants were tested for their effects on both HR and NHEJ. The HR assay consists in the evaluation of the frequency of Hyg^R clones due to the cell's ability to reconstitute the wild-type *Hyg* gene that is located, in two mutated copies, in the vector pTPSN stably integrated in the cell genome.

The NHEJ assay consists in the evaluation of the frequency of puromycin-resistant clones due to random chromosomal integration of a plasmid DNA containing the puromycin-resistance gene (Fig. 3).

As shown in our experimental conditions, *BRCA1 wild type* increases the HR frequency. Moreover, none of the BRCA1-UCVs altered the HR frequency when compared to the *BRCA1 wild type*. As a matter of fact, the low increase in HR frequency obtained when the *BRCA1 wild type* was over-expressed, even if statistically significant, could not be biologically relevant. A twofold increase in HR frequency has been proposed as cut-off value to be considered as biologically relevant [28, 29]. In our experiments, no BRCA1 missense variant increased HR by twofold; therefore, we can conclude that this assay does not distinguish between pathogenic mutation and neutral polymorphism. Recently, we have developed a yeast-recombination assay that could be helpful to characterize BRCA1 missense variants [30]. In yeast, the over-expression of pathogenic BRCA1 variants induce HR by twofold to fourfold as compared to the wild-type or neutral polymorphism [30] (Table 3). Thus, the yeast, *Saccharomyces cerevisiae*, assay is able to distinguish the pathogenic from the neutral BRCA1 missense variants. So far, we could not exactly understand this different effect of the BRCA1 variants on yeast HR as compared to HeLa cells (Table 3); the ratio between NHEJ and HR varies greatly across phylogenetic groups. Yeast relies heavily on HR, while in mammals and plants, NHEJ is the preferred pathway. The choice may be dictated by genome composition. In large repetitive genomes of plants and animals, overly efficient HR may lead to deleterious genomic rearrangements, such that NHEJ may be a safer choice [31]. This is the main reason why we measured the effect of BRCA1 missense variants on NHEJ in a plasmid random integration assay. Notably, BRCA1 was shown to be involved also in the regulation of random integration by NHEJ, even when the molecular mechanism has not been fully understood [32]. Different kinds of assays support this involvement such as in vitro reconstitution of a linearized plasmid, in vivo overall end-joining and microhomology mediated end-joining [12, 33].

The results of this study confirmed a clear involvement of BRCA1 in random chromosomal integration of a linearized plasmid DNA. The over-expression of BRCA1-Y179C and BRCA1-A1789T UCVs increased the frequency of random integration as compared to the wild type. It was observed that the over-expression of BRCA1-Y179C induces a hyper-recombination phenotype also in yeast (Table 3) [30]. Moreover, we have previously reported that the in vivo analysis on tumor tissue revealed that the proband carrier of the Y179C showed loss of heterozigosity (LoH) of the wild-type allele, and the proband carrier of A1789T showed hypermethylation of the wild-type allele. Both LoH and hypermethylation are considered to be indicative of the pathogenicity of the variant [30].

The UCVs, I1766S and N550H, did not affect the NHEJ frequency, as well as the pathogenetic control M1775R, suggesting that their roles are not related to the NHEJ pathway. However, both the I1766S and the mutation M1775R affected the transcriptional activation ability of BRCA1 both in yeast and mammalian cells [21]. The A1789T variant also, in addition to its effect in the NHEJ assay, appeared to abrogate the BRCA1 transcriptional activity (L. Guidugli unpublished result), suggesting its potentially pathogenic characteristic.

These findings suggest that the BRCA1 protein may have completely independent functions related to specific protein regions. In terms of defining the influence of UCVs on BRCA1 function, these findings indicate that all UCVs should be analyzed by all the functional methods available. If only one assay is used, then it is possible that a UCV that inactivates a different function of BRCA1 might be identified as having no clinical relevance.

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Conflicts of interest None.

References

1. Chen J, Silver DP, Walpita D, Cantor SB, Gazdar AF, Tomlinson G, Couch FJ, Weber BL, Ashley T, Livingston DM, Scully R (1998) Stable interaction between the products of the BRCA1 and BRCA2 tumor suppressor genes in mitotic and meiotic cells. Mol Cell 2(3):317–328. doi:S1097-2765(00)80276-2
2. Scully R, Chen J, Plug A, Xiao Y, Weaver D, Feunteun J, Ashley T, Livingston DM (1997) Association of BRCA1 with RAD51 in mitotic and meiotic cells. Cell 88(2):265–275. doi:S0092-8674(00)81847-4[pii]
3. Wu W, Koike A, Takeshita T, Ohta T (2008) The ubiquitin E3 ligase activity of BRCA1 and its biological functions. Cell Div 3:1. doi:10.1186/1747-1028-3-1
4. Goldgar DE, Easton DF, Deffenbaugh AM, Monteiro AN, Tavtigian SV, Couch FJ (2004) Integrated evaluation of DNA sequence variants of unknown clinical significance: application to BRCA1 and BRCA2. Am J Hum Genet 75(4):535–544. doi:10.1086/424388S0002-9297(07)62706-2
5. Abkevich V, Zharkikh A, Deffenbaugh AM, Frank D, Chen Y, Shattuck D, Skolnick MH, Gutin A, Tavtigian SV (2004) Analysis of missense variation in human BRCA1 in the context of interspecific sequence variation. J Med Genet 41(7):492–507
6. Mirkovic N, Marti-Renom MA, Weber BL, Sali A, Monteiro AN (2004) Structure-based assessment of missense mutations in human BRCA1: implications for breast and ovarian cancer predisposition. Cancer Res 64(11):3790–3797. doi:10.1158/0008-5472.CAN-03-300964/11/3790[pii]
7. Couch FJ, Rasmussen LJ, Hofstra R, Monteiro AN, Greenblatt MS, de Wind N, IARC Unclassified Genetic Variants Working Group (2008) Assessment of functional effects of unclassified genetic variants. Hum Mutat 29(11):1314–1326
8. Deng CX (2006) BRCA1: cell cycle checkpoint, genetic instability, DNA damage response and cancer evolution. Nucleic Acids Res 34(5):1416–1426. doi:34/5/141610.1093/nar/gkl010
9. Hakem R, de la Pompa JL, Sirard C, Mo R, Woo M, Hakem A, Wakeham A, Potter J, Reitmair A, Billia F, Firpo E, Hui CC,

Roberts J, Rossant J, Mak TW (1996) The tumor suppressor gene BRCA1 is required for embryonic cellular proliferation in the mouse. Cell 85(7):1009–1023. doi:S0092-8674(00)81302-1

10. Zhong Q, Chen CF, Li S, Chen Y, Wang CC, Xiao J, Chen PL, Sharp ZD, Lee WH (1999) Association of BRCA1 with the hrad50-hmre11–p95 complex and the DNA damage response. Science 285(5428):747–750
11. Bau DT, Fu YP, Chen ST, Cheng TC, Yu JC, Wu PE, Shen CY (2004) Breast cancer risk and the DNA double-strand break end-joining capacity of nonhomologous end-joining genes are affected by BRCA1. Cancer Res 64(14):5013–5019
12. Zhong Q, Chen CF, Chen PL, Lee WH (2002) BRCA1 facilitates microhomology-mediated end joining of DNA double strand breaks. J Biol Chem 277(32):28641–28647. doi:10.1074/jbc.M200748200M200748200
13. Snouwaert JN, Gowen LC, Latour AM, Mohn AR, Xiao A, DiBiase L, Koller BH (1999) BRCA1 deficient embryonic stem cells display a decreased homologous recombination frequency and an increased frequency of non-homologous recombination that is corrected by expression of a BRCA1 transgene. Oncogene 18(55):7900–7907. doi:10.1038/sj.onc.1203334
14. Ng PC, Henikoff S (2003) SIFT: predicting amino acid changes that affect protein function. Nucleic Acids Res 31(13):3812–3814
15. Ramensky V, Bork P, Sunyaev S (2002) Human non-synonymous snps: server and survey. Nucleic Acids Res 30(17):3894–3900
16. Tavtigian SV, Deffenbaugh AM, Yin L, Judkins T, Scholl T, Samollow PB, de Silva D, Zharkikh A, Thomas A (2006) Comprehensive statistical study of 452 BRCA1 missense substitutions with classification of eight recurrent substitutions as neutral. J Med Genet 43(4):295–305. doi:jmg.2005.033878[pii]10.1136/jmg.2005.033878
17. Ciotta C, Ceccotti S, Aquilina G, Humbert O, Palombo F, Jiricny J, Bignami M (1998) Increased somatic recombination in methylation tolerant human cells with defective DNA mismatch repair. J Mol Biol 276(4):705–719. doi:S0022-2836(97)91559-X[pii]10.1006/jmbi.1997.1559
18. Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C (2006) Clonogenic assay of cells in vitro. Nat Protoc 1(5):2315–2319. doi:nprot.2006.339[pii]10.1038/nprot.2006.339
19. Di Primio C, Galli A, Cervelli T, Zoppe M, Rainaldi G (2005) Potentiation of gene targeting in human cells by expression of saccharomyces cerevisiae rad52. Nucleic Acids Res 33(14):4639–4648. doi:33/14/4639[pii]10.1093/nar/gki778
20. Williams RS, Glover JN (2003) Structural consequences of a cancer-causing BRCA1-brct missense mutation. J Biol Chem 278(4):2630–2635. doi:10.1074/jbc.M210019200M210019200
21. Carvalho MA, Marsillac SM, Karchin R, Manoukian S, Grist S, Swaby RF, Urmenyi TP, Rondinelli E, Silva R, Gayol L, Baumbach L, Sutphen R, Pickard-Brzosowicz JL, Nathanson KL, Sali A, Goldgar D, Couch FJ, Radice P, Monteiro AN (2007) Determination of cancer risk associated with germ line BRCA1 missense variants by functional analysis. Cancer Res 67(4):1494–1501. doi:67/4/1494[pii]10.1158/0008-5472.CAN-06-3297
22. Judkins T, Hendrickson BC, Deffenbaugh AM, Eliason K, Leclair B, Norton MJ, Ward BE, Pruss D, Scholl T (2005) Application of embryonic lethal or other obvious phenotypes to characterize the clinical significance of genetic variants found in trans with known deleterious mutations. Cancer Res 65(21):10096–10103. doi:65/21/10096[pii]10.1158/0008-5472.CAN-05-1241
23. Goldgar DE, Easton DF, Byrnes GB, Spurdle AB, Iversen ES, Greenblatt MS (2008) Genetic evidence and integration of various data sources for classifying uncertain variants into a single model. Hum Mutat 29(11):1265–1272. doi:10.1002/humu.20897
24. Tavtigian SV, Greenblatt MS, Goldgar DE, Boffetta P (2008) Assessing pathogenicity: overview of results from the IARC Unclassified Genetic Variants Working Group. Hum Mutat 29(11):1261–1264. doi:10.1002/humu.20903
25. Chenevix-Trench G, Healey S, Lakhani S, Waring P, Cummings M, Brinkworth R, Deffenbaugh AM, Burbidge LA, Pruss D, Judkins T, Scholl T, Bekessy A, Marsh A, Lovelock P, Wong M, Tesoriero A, Renard H, Southey M, Hopper JL, Yannoukakos K, Brown M, Easton D, Tavtigian SV, Goldgar D, Spurdle AB (2006) Genetic and histopathologic evaluation of BRCA1 and BRCA2 DNA sequence variants of unknown clinical significance. Cancer Res 66(4):2019–2027. doi:66/4/2019[pii]10.1158/0008-5472.CAN-05-3546
26. Venkitaraman AR (2009) Linking the cellular functions of BRCA genes to cancer pathogenesis and treatment. Annu Rev Pathol 4:461–487. doi:10.1146/annurev.pathol.3.121806.151422
27. Phelan CM, Dapic V, Tice B, Favis R, Kwan E, Barany F, Manoukian S, Radice P, van der Luijt RB, van Nesselrooij BP, Chenevix-Trench G, ConFab K, Caldes T, de la Hoya M, Lindquist S, Tavtigian SV, Goldgar D, Borg A, Narod SA, Monteiro AN (2005) Classification of BRCA1 missense variants of unknown clinical significance. J Med Genet 42(2):138–146. doi:42/2/138[pii]10.1136/jmg.2004.024711
28. Galli A, Schiestl RH (1996) Effects of salmonella assay negative and positive carcinogens on intrachromosomal recombination in G1-arrested yeast cells. Mutat Res 370(3–4):209–221
29. Galli A, Schiestl RH (1995) Salmonella test positive and negative carcinogens show different effects on intrachromosomal recombination in G2 cell cycle arrested yeast cells. Carcinogenesis 16(3):659–663
30. Caligo MA, Bonatti F, Guidugli L, Aretini P, Galli A (2009) A yeast recombination assay to characterize human BRCA1 missense variants of unknown pathological significance. Hum Mutat 30(1):123–133. doi:10.1002/humu.20817
31. Mao Z, Bozzella M, Seluanov A, Gorbunova V (2008) Comparison of nonhomologous end joining and homologous recombination in human cells. DNA Repair 7(10):1765–1771
32. Zhong Q, Boyer TG, Chen PL, Lee WH (2002) Deficient nonhomologous end-joining activity in cell-free extracts from BRCA1-null fibroblasts. Cancer Res 62(14):3966–3970
33. Baldeyron C, Jacquemin E, Smith J, Jacquemont C, De Oliveira I, Gad S, Feunteun J, Stoppa-Lyonnet D, Papadopoulo D (2002) A single mutated BRCA1 allele leads to impaired fidelity of double strand break end-joining. Oncogene 21(9):1401–1410. doi:10.1038/sj.onc.1205200