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A recombination-based method to characterize human BRCA1 missense variants

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Abstract

Purpose. 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 the present study was to evaluate BRCA1 missense variants for their effect on both Homologous Recombination (HR) and Non Homologous End Joining (NHEJ).

Methods. HR frequency evaluation: HeLaG1 cells, containing a stably integrated plasmid that allows to measure HR events by gene conversion events were transfected with the pcDNA3 β expression vector containing the BRCA1-wild type (BRCA1-WT) or the BRCA1-Unclassified Variants (BRCA1-UCVs).

The NHEJ was measured by a random plasmid integration assay.

Results. This assays suggested a BRCA1 involvement mainly in the NHEJ. As a matter of fact, the Y179C and the A1789T variant altered significantly 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. **Conclusions.** These data, beside 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 in order to identify variants with altered function.

Keywords: Homologous Recombination, Non Homologous End Joining, Unclassified Variants, BRCA1, breast cancer, functional assay.

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 that 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 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 that 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 in order 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 been yet completely clarified. As a matter of fact the frequency of random plasmid integration in transiently *BRCA1*-wt transfected HCC1937 cells is significantly increased as compared to the parental cell line [13] whereas this phenomenon is also impaired in *BRCA1*^{-/-} mouse embryonic fibroblasts but contradictory results were obtained [10] [11,12].

In this study, we selected some missense variants from a mutational screening of 276 breast and/or ovarian cancer families. Four non synonymous variants, that localized in different BRCA1 functional domains, were identified as potentially deleterious and likely disrupting the gene function by 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 overexpression of the wild type or these mutated BRCA1 protein on spontaneous HR and NHEJ events in HeLa cells.

MATERIALS & METHODS

SAMPLES AND MUTATION SELECTION

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; 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 in order to optimise 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 BRCA1wt 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 line was 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% CO₂ 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 was ranging from 70-85%.

IMMUNOBLOTTING

Twenty four hours after transfection of pcDNA3BRCA1, aliquots of 4×10^5 cells were washed twice in Phosphate Buffered Saline (PBS) 1X and lysed in the Laemmli Sample Buffer 1X (Tris-HCl 50 Mm pH 6.8, SDS 2%, glycerol 10%, bromophenol blue 0.1 %, β -mercaptoethanol 100mM) together with the Protease Inhibitor Cocktail 1X (Sigma). The protein extracts were denaturated at 100°C for 5 minutes. A total of ~ 120 μ g of whole cell extract was subjected to electrophoresis at 10 to 20 mA for ~ 3 hours in a 6% SDS-polyacrylamide gel; thereafter, the proteins were transferred to polyvinylidene fluoride membrane at 170 mA for 17 hours 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 secondary antibody. The BRCA1 protein was detected using the ECL chemiluminescence 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 *PvuI* site (*hyg1*), the other Hyg^R at the *SacII* site (*hyg2*) (Figure 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 x 10⁻⁵ viable cells. HeLaG1 cells were transfected with the pcDNA3β expression vector containing the wild type *BRCA1* or the *BRCA1*-UCVs. 24 hours after transfection, cells were harvested and plated (6x10⁵ cells/10 cm dish and 10² cells/6cm dish, for plating efficiency (PE) evaluation). For the selection of the recombination events, 24 hours later we added hygromycin 0.2 mg/ml (Sigma) to the medium. Medium was changed twice and, after 10 to 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 2μg of the pcDNA3 .BRCA1-wt or BRCA1-UCV vectors and 2μg of pBlue-puro that carries no homology with the genome of HeLa cells and, therefore, it stably integrates by non homologous recombination[19]. One day after transfection, cells were collected and plated (2 x 10⁵ 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 analysed by the t- Student Test. All the analysis were performed by using Statgraphics (StatPoint Inc.USA).

RESULTS

Variants Selection

We selected four non synonymous UCVs suggested by Sift, Polyphen and Align-GVGD software as likely disrupting the protein function: the Y179C, the N550H, the I1766S and the A1789T (Table 1 and Figure 1) identified in four 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 (figure 2a). The I1766S was classified as a deleterious amino acidic change by Carvalho et al. [21]. It was found in one family: 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 (figure 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 (figure 2c-d). 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 breast cancer at 48 and 53 years of age. The proband's mother and two cousins were affected by breast cancer. Unfortunately no one 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 -BRCA1-wt, 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, Figure 3). First, we checked if the expression of the wild type and mutated *BRCA1* was detectable 24 hours after transfection. Then, we prepared the total lysate, as described in the methods, and carried out Western blot analysis. In the figure 4, we showed 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 (figure 4). The expression of endogenous BRCA1 was seen only after 2 hours of exposure (data not shown). Thus, under these conditions, we concluded that the exogenous BRCA1 proteins were over-expressed. This prompted us to determine if this transient expression of the BRCA1 protein affected recombination. For such reason, 24 hours after transfection the cells were seeded in the presence of hygromycin 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 $P < 0.005$) (Table2): 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 homologous recombination in human cells would be presumably not helpful to characterize BRCA1 UCVs.

Random plasmid integration in HeLa cells

To evaluate whether BRCA1 UCVs had an influence on NHEJ, we determined the effect of

the expression of these proteins on random (non-homologous) plasmid integration in HeLa cells. The plasmid expressing the *BRCA1* wt or *BRCA1* UCVs was co-transfected with the pBlue-puro plasmid; after 24 hours, 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-expression 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). 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 wt, 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 remains 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 a tumor suppressor gene and germ-line mutations which disrupt its functions culminate, after the loss of the wild type allele, in cancer development. 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 not comprehensive functional assay available for BRCA1 [27,28]. In this paper, 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 effect on both HR and NHEJ. The HR assay consists in the evaluation of the frequency of Hyg^R clones due to the cell 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 on random chromosomal integration of a plasmid DNA containing the puromycin-resistance gene (Figure 3).

We showed that in our experimental conditions, BRCA1-WT increases the HR frequency. Moreover, none of the BRCA1-UCVs altered the HR frequency when compared to the BRCA1-WT. As a matter of fact, the low increase in HR frequency obtained when the BRCA1-WT was over-expressed, even if statistically significant, could be not biologically relevant. A 2 fold increase in HR frequency has been proposed as cut-off value to be considered as biologically relevant [29,30]. In our experiments, no BRCA1 missense variant increased HR by 2 fold, 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 [31]. In yeast, the over-expression of pathogenic BRCA1 variants induce HR by 2-4 fold as compare to the wild type or neutral polymorphism [31](Table 3).

Thus, the yeast *Saccharomyces cerevisiae* assay is able to distinguish the pathogenic from the neutral BRCA1 missense variants. So far, we do 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 rely 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 [32]. 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 if the molecular mechanism has not fully understood [33]. 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 [11,34].

Our results 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) [31]. Moreover, we have previously reported that the *in vivo* analysis on tumor tissue revealed that the proband carrier of the Y179C showed loss of heterozygosity (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 [31].

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, showed to abrogate the BRCA1 transcriptional activity (Guidugli unpublished results), suggesting to be potentially pathogenic.

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, it is possible that a UCV that inactivates a different function of BRCA1 might be identified as having no clinical relevance.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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FIGURE LEGENDS

Fig. 1 Localization of the UCVs in the BRCA1 cDNA sequence. The numbers indicate the exons.
*Mutation localized in BRCT Domain; #Pathogenetic control variant

Fig. 2 Pedigrees of families harboring the variants A1789T (2a), I1766S (2b), Y179C and N550H (3a)

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 *PvuI* site (*hyg1*) or at a unique *SacII* 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 wt or UCVs

Fig. 4 Western Blot analysis to measure the expression of the BRCA1 wt 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-BRCA1-A1789T; 5) pcDNA3-BRCA1-I1766S; 6) pcDNA3-BRCA1-Y179C; 7) pcDNA3-BRCA1-N550H.