

# Missense mutations of *BRCA1* gene affect the binding with p53 both *in vitro* and *in vivo*

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**Abstract.** Women with *BRCA1* gene mutations have an increased risk for breast and ovarian cancer (BOC). Classification of missense variants as neutral or disease causing is still a challenge and has major implications for genetic counseling. *BRCA1* is organized in an N-terminal ring-finger domain and two BRCT (breast cancer C-terminus) domains, involved in protein-protein interaction. The integrity of the C-terminal, BRCT repeat region is also critical for *BRCA1* tumor suppressor function. Several molecular partners of *BRCA1* have so far been identified; among them, the tumor suppressor protein p53 seems to play a major role. This study was aimed at evaluating the impact of two missense mutations, namely the W1837R and the S1841N, previously identified in BOC patients and located in the BRCT domain of the *BRCA1* gene, on the binding capacity of this protein to p53. Co-immunoprecipitation assays of *E. coli*-expressed wild-type and mutated BRCTs challenged with a HeLa cell extract revealed, for the S1841N variant a significant reduction in the binding activity to p53, while the W1837R mutant showed an inverse effect. Furthermore, a clonogenic soft agar growth assay performed on HeLa cells stably transfected with either wild-type or mutant *BRCA1* showed a marked decrease of the growth in wild-type *BRCA1*-overexpressing cells and in *BRCA1*<sup>S1841N</sup>-transfected cells, while no significant changes were detected in the *BRCA1*<sup>W1837R</sup>-transfected cells. These results demonstrate that: i) distinct single nucleotide changes in the BRCT domain of *BRCA1* affect binding of this protein to the tumor suppressor p53, and ii) the two missense mutations here described are likely to play a role in breast tumorigenesis. We suggest that *in vitro/in vivo* experiments testing the effects of unclassified

*BRCA1* gene variants should therefore be taken in to consideration and that increased surveillance should be adopted in individuals bearing these two *BRCA1* missense alterations.

## Introduction

*BRCA1* is a tumor suppressor gene whose germ-line mutations predispose to breast and ovarian cancer (1,2). *BRCA1* encodes a nuclear phosphoprotein of about 220 Kd, functionally organized in domains located at the N- and C-terminus of the molecule (3). In particular, the COOH-terminal BRCT domain is an evolutionarily conserved region characterized by hydrophobic clusters of amino acids that are thought to stabilize the three-dimensional structure of the protein (4). Along with the role in the stability of protein conformation, the BRCT domain is involved in protein-protein interactions with many different molecules (5). Several *BRCA1* partners have been so far identified, including components of the DNA repair machinery (6), transcription factors (7-9), the transcriptional co-activator p300 (10) and p53 (11-13). Binding of *BRCA1* to p53 requires a two-domain interaction, located at the C-terminus (aa 1756-1855), and at the N-terminus (aa 224-500) (11), respectively.

p53 is a nuclear protein of about 53 kDa in size (14,15), subdivided into four domains with distinct but interdependent functions (16,17). Several triggers can activate p53 that, in turn, modulates a set of genes with protective activities for the cell, including those involved in control of the cell cycle and induction of apoptosis (17). The promoters of *p21* and *Bax* genes are some of these p53-activated targets. The physical association of p53 with *BRCA1* increases p53-dependent transcription driven by these two promoters (18,19). Mutations in the coding region of p53 have been identified in approximately 30-50% of breast cancer (20). Mutated p53 can no longer regulate *BRCA1* expression in mammary tissue. Loss of either p53 or *BRCA1* function leads to disruption of cell cycle check-points, accumulation of DNA damage, and ultimately to sporadic breast cancer (21).

It has been estimated that *BRCA1* gene mutations account for about 50% of hereditary breast cancer (BC) and for about 80% of hereditary breast and ovarian cancer (BOC) (22). Interestingly, the majority of known cancer-causing *BRCA1* mutations are localized in the N- and in the C-terminal interaction domains (2,23-26). While the pathogenic role of non-sense and frame shift mutations is well recognized in breast

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carcinogenesis, the impact of missense mutations is still to be defined.

The aim of our study was to evaluate the role played by two distinct missense mutations in the BRCT domain of the *BRCA1* gene on the binding of this protein to the partner p53 and to shed more light on their pathogenic role in breast carcinogenesis. Both mutations have been detected in BC and/or BOC cancer patients.

Wild-type and mutated BRCTs have been expressed in *E. coli*, challenged with a HeLa cell extract used as a source of p53 and then analyzed by Western blotting with an anti-p53 antibody. The results of the co-immunoprecipitation assay show a significant reduction in the binding to p53 by the BRCT domain carrying the S1841N amino acid change (of the order of 72%), while the BRCT<sup>W1837R</sup> mutation potentiates binding of BRCA1 to p53.

To further investigate the biological effects of *BRCA1* missense mutations on the cellular phenotype, a clonogenic soft agar growth assay has been performed on HeLa cells stably transfected with wild-type and mutant *BRCA1* genes. Interestingly, wild-type BRCA1-overexpressing cells show a marked decrease of the clonogenic growth, while the BRCA1<sup>W1837R</sup> transfected cells have a markedly different behavior, mimicking the untransfected HeLa cells. On the other hand, a significant reduction of cell growth is found in BRCA1<sup>S1841N</sup> transfected cells.

Our results provide new insights into the molecular mechanisms underlying the pathogenic role of single nucleotide mutations on *BRCA1*-dependent BC tumorigenesis and may be considered a valuable approach in the identification of genetic alterations potentially critical in the predisposition of individuals to cancer.

## Materials and methods

**Cell culture and transfection.** HeLa cells were cultured as monolayer in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% (v/v) fetal calf serum (Sigma), 100 units/l penicillin (Hyclone). Cells were grown at 37°C in a 5% CO<sub>2</sub> atmosphere. Parental HeLa cells were transfected with pcDNA 3.1 (empty vector transfected), pcDNA BRCA1wt, pcDNA BRCA1 W1837R and pcDNA BRCA1 S1841N using the calcium phosphate precipitation method. Cells were then selected in G418 400 µg/ml (Invitrogen, Life Technologies).

**Plasmids.** The pcDNA3.1 plasmid containing the full-length *BRCA1* gene (pcDNA BRCA1wt) was a gift of Dr M. Montagna. Site-directed mutagenesis of the *BRCA1* cDNA insert was obtained using the QuickChange Kit (Stratagene). The *BRCA1* mutants were generated with the following primers: forward 5'-GACCCGAGAGCGGGTGTGGACAGTG-3' and reverse 5'-CACTGTCCAACACCCGCTCTCGGGTC-3' for pcDNA BRCA1<sup>W1837R</sup>; forward 5'-GTGTTGGA CAATGTAGCACTCTACC-3' and reverse 5'-GGTAGAGT GCTACATTGTCCAACAC-3' for pcDNA BRCA1<sup>S1841N</sup>.

The wild-type and mutant *BRCA1*s encoding the region from aa 1537 to aa 1861 were generated with the following primers: forward 5'-GCCCGGGATGCAACAGCTGGAAGAG-3' and reverse 5'-AAGCTTTTAGCTGGGGATCTGGG-3'

and cloned into the *SmaI* site of pQE-30 (Qiaexpress Kit, Promega). All constructs were verified by DNA sequencing. The DH5α strain was used in cloning procedures.

**p53-BRCA1 interaction in vitro.** *E. coli* strains M15 (pRep4) harboring pQE BRCA1<sup>wt</sup>, pQE BRCA1<sup>W1837R</sup> and pQE BRCA1<sup>S1841N</sup> were grown to early logarithmic phase. After incubation with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h at 37°C, bacteria were lysed by sonication in buffer H (20 mM Hepes; 300 mM KCl; 0.1 mM EDTA; 0.1% NP40; 1 mM PMSF), and protein products were purified from the soluble extract fraction by centrifugation. The supernatants were incubated with Ni-NTA agarose for 2 h at 4°C. Histidine-tagged proteins were collected by centrifugation, washed four times with buffer H and incubated for 2 h at 4°C with 1 mg of HeLa cell total extract (27). The beads were collected by centrifugation and washed four times with buffer H. The proteins were electrophoresed on SDS/10%-PAGE gels and transferred to a nitrocellulose filter (Immobilon<sup>TM</sup> P; Millipore). After addition of the blocking mixture [5% (w/v) BSA in PBS, pH 7.4], the membrane was incubated with a 1:200 dilution of rabbit anti-human BRCA1 antibody (Santa Cruz) or of mouse anti-human p53 antibody (Santa Cruz) for 2 h at room temperature. Bound antibody was detected with anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5000) or anti-mouse horseradish peroxidase-conjugated secondary antibody (1:5000), respectively. Following three washes in PBS-Tween for 10 min each time, the membrane was developed by ECL-Western blot detection reagents according to the manufacturer's instructions (Santa Cruz).

**Soft agar growth assay.** Soft agar assay was performed using 5x10<sup>4</sup> HeLa parental cells, HeLa/pcDNA 3.1, HeLa/<sup>wt</sup>BRCA1, HeLa/<sup>S1841N</sup>BRCA1 and HeLa/<sup>W1837R</sup>BRCA1 respectively, assayed for BRCA1 protein expression. They were suspended in 1.5 ml of 0.5% Noble Agar (Difco, Kansas City, MO, USA), 100 mM triptose phosphate buffer (Difco), 10% FCS DMEM medium and layered on 7 ml of similar buffer in 60-mm culture dishes. Plates were incubated at a constant temperature of 37°C with a humidified atmosphere of 5% CO<sub>2</sub> for 3 weeks and counted.

## Results

**Effects of BRCT missense mutations on the interaction with p53.** The BRCT domain is a main target for cancer associated mutations; out of 891 (28) DNA variants so far reported in the *BRCA1* gene more than 22% are localized within this region. The BRCT domain is also one of the two domains responsible for the binding of BRCA1 with p53 (11). In order to assess the potential impact of BRCT missense mutations to the binding with the partner protein p53, two distinct variants (W1837R and S1841N) were introduced in the wild-type BRCT domain of *BRCA1* cDNA by site-directed mutagenesis (Fig. 1). The wild-type and the mutagenized cDNA fragments (from nt 4611 to nt 5583), cloned in the His-tag pQE30 plasmid, were then expressed in *E. coli*, immobilized on Ni-NTA Agarose and used as bait against a total protein extract from HeLa cells transfected with a p53 expression vector. After extensive washing, p53 protein interacting with

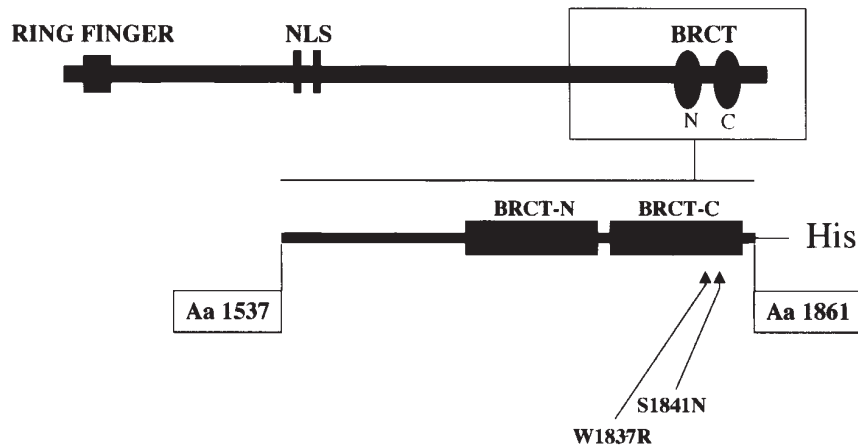


Figure 1. Domain structure of BRCA1. (Top), Schematic representation of full-length BRCA1 protein featuring the RING domain in the N-terminal region and BRCT domains in the C-terminal region. The region analyzed (aa 1537-1863) is contained in the box, which is enlarged and presented (bottom), BRCA1 C-terminal region (aa 1537-1863) His tagged. Mutations analyzed are depicted.

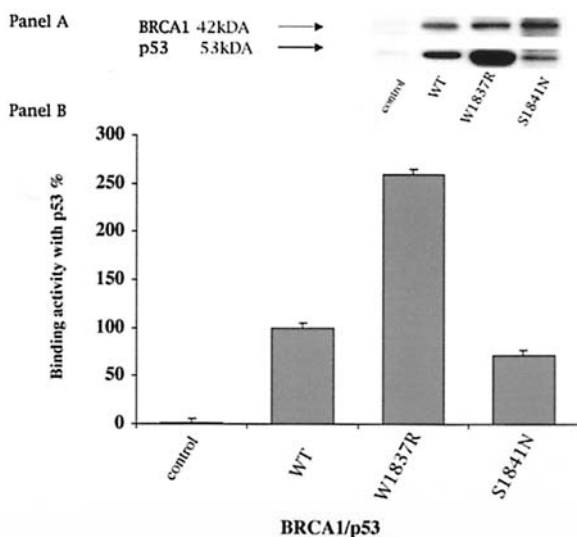


Figure 2. Interaction of p53 with histidine-tagged BRCA1. Panel A, Amounts of histidine-tagged wild-type (wt) or mutated BRCA1 proteins ('W1837R' and 'S1841N') were immobilized on Ni-NTA-agarose (upper panel), and challenged with total protein extract derived from HeLa cells as described in Materials and methods. After extensive washing, the amount of p53 protein interacting with wild-type or mutated BRCA1 proteins was determined by Western blot using a mouse anti-human p53 antibody (lower panel). The expected molecular masses of BRCA1 and p53 proteins are indicated on the left. Panel B, Quantitative analysis of the interaction between p53 protein and histidine-tagged wild-type or mutated BRCA1 proteins. Values are means for at least five independent experiments shown in (A) and they represent percentages ( $\pm$ SEM) of densitometry values.

BRCA1 was assayed by Western blotting using a rabbit anti-p53 antibody, as shown in Fig. 2. Because the expression efficiency of mutant BRCA1 polypeptides in bacterial cells was not homogeneous, protein concentration in the immunoprecipitation assay was adjusted to contain equivalent amounts of BRCA1, as estimated by Western blot performed with a mouse anti-His-tag antibody (Fig. 2, panel A). The empty pQE30 vector (Fig. 2, panel A) and the untreated Ni-NTA agarose (data not shown), were used as negative controls. The relative intensity of the signal was quantified by densitometry scanning of the filter (Fig. 2, panel B). The results

indicate that the two missense mutations in the BRCT domain do not abolish the binding of BRCA1 to p53, but are able to consistently alter the heterodimer complex formation. More specifically, in the S1841N allele, the reduced intensity of the chemiluminescence signal, compared to that of the wild-type allele, strongly suggests a defect in binding to p53, while the W1837R replacement consistently increases the interaction with p53 (on average about 2.5-fold), and/or the stability of the protein complex.

*Effects of BRCA1 variants on colony formation in soft agar.* The biochemical analysis of BRCTs mutant alleles indicates that nucleotide substitutions in BRCT domain modulate BRCA1/p53 interactions. Since both p53 and BRCA1 are related with control of cell growth and neoplastic transformation (29), we decided to test the growth properties of the mutant alleles by measuring their ability to form colonies in a soft agar assay.

To this end, we stably transfected HeLa cells with either the wild-type *BRCA1* cDNA (HeLa/<sup>wt</sup> BRCA1) or with the W1837R (HeLa/<sup>W1837R</sup> BRCA1) and S1841N (HeLa/<sup>S1841N</sup> BRCA1) mutated forms (Materials and methods). HeLa cells transfected with vector alone were used as control. The clones were selected with G-418 and the expression efficiency of wild-type and mutant BRCA1 His-tagged proteins was checked by Western blot analysis (data not shown). HeLa cells were subsequently layered on soft agar (Materials and methods) and the colonies were stained and counted after 3 weeks. Striking differences were observed in two independent selection experiments, both in number (Fig. 3) and size (data not shown) of colonies formed. The BRCA1 wild-type expressing clone produced a reduced number of colonies (about 30%) compared to the ones generated by parental HeLa; approximately the same number of colonies was obtained with the S1841N allele (about 28% of the control). The size of the S1841N colonies was consistently smaller than the size of colonies expressing wt BRCA1 and parental control HeLa. On the other hand, the W1837R-expressing clone produced colonies comparable in size and number to those produced by parental HeLa. These experiments indicate that the expression of wt BRCA1 reduces the ability of HeLa cells to grow on soft



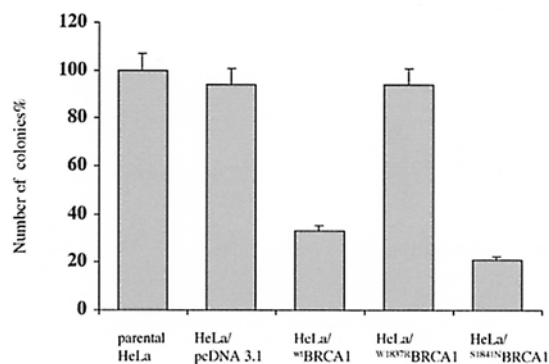


Figure 3. Soft agar growth assay. Quantitative analysis of *in vivo* clonogenic growth assay on HeLa cells stably transfected with either the wild-type BRCA1 cDNA (HeLa/<sup>wt</sup> BRCA1) or two distinct mutagenized BRCA1 cDNA (HeLa/<sup>W1837R</sup> BRCA1 and HeLa/<sup>S1841N</sup> BRCA1) (Materials and methods). Values are means for at least two independent experiments.

agar, as it does for expression of the S1841N allele. Conversely, the expression of the W1837R allele fully restores the ability of HeLa cells to grow without adhering to a solid matrix, to an extent comparable to that of parental cells.

## Discussion

This study analyzed the potential role of *BRCA1* missense mutations identified in families with hereditary BC and BOC. While the pathogenic role of non-sense and frame shift germ line mutations is well recognized in BC/BOC carcinogenesis, the impact of missense mutations, accounting for about 37% (28) of the overall *BRCA1* gene alterations, is still to be defined. This latter type of mutations, resulting solely in the substitution of a single aa residue, do not cause premature termination of the polypeptide but might alter critical physico-chemical properties potentially responsible for the stabilization the BRCA1 protein structure and/or its interactions with other proteins, thereby impairing the normal function and predisposing to BC.

Several approaches have been used to characterize missense variants at the BRCT domains. Williams *et al* (30) used a protease-based assay to directly assess the sensitivity of the folding of the BRCT domain to a set of truncations as well as single amino acid substitutions derived from BC screening programs; Gaiser *et al* (31) performed dynamics measurements on the NMR structure of the isolated C-terminal BRCT domain of human BRCA1; Mirkovic *et al* (32) made a structure-based assessment of missense mutations in human *BRCA1*, with the specific aim to increase the ability to identify cancer-associated variants, particularly those that abolish transcriptional activation.

In this report, we have analyzed the role of two missense mutations in the BRCT domain with respect to two parameters, i.e. the ability to bind p53 and the capacity in forming colonies in a soft agar assay. The results of protein-protein interaction analysis indicate that the *BRCA1* allele carrying the S1841N mutation significantly reduces binding to p53 when compared with the wt allele, while the W1837R modification produces an inverse effect. Thus, mutations affecting strictly contiguous amino acids may lead to opposite biological effects.

The crystal structure of rat BRCA1 tandem-BRCT region (PDB 1L0B) has been resolved (<http://www.rcsb.org/pdb>) (33). In this model, the BRCT domain can be subdivided in three distinct fragments [fragment 1, from ARG 1591 to GLN 1702 (112 aa); fragment 2, from LEU 1705 to GLU 1756 (52 aa); and fragment 3, from ARG 1776 to GLN 1801 (26 aa) and in three loop areas (L1a from 1688 to 1693; L1b from 1638 to 1641; L3 from 1788 to 1801)]. The loops 1a and 1b are known to be involved in the interaction with p53 (33). The rat and human amino acid sequences show a high degree (about 70%) of homology (30). In the sequence alignment, the human W1837 corresponds to the rat W1782 and the S1841 corresponds to the rat S1786. The W1782 residue, that is localized exactly in the middle of an  $\alpha$  helix, shows a consistent degree of interaction with ASP1794 side chain, as well as with other amino acids, such as VAL1778, PHE1706 and VAL1783, located within a hydrophobic cleft. We suggest that the substitution of a non-polar aromatic side-chain with the basic amino acid ARG, directly in contact with the terminal loop L3, may dramatically modify the network of H-bonds in this segment of the BRCA1 molecule. As a consequence, important conformational changes may take place in the terminal residues of the L3 loop, and produce an increase in its conformational rigidity that may strengthen the binding with p53 and/or the stability of the protein complex. Concerning the SER1786, the OH group of its side chain interacts with TRP1782 and with ARG1793 with H-bonds and the amidic H interacts with VAL1783 residue, stabilizing the conformation of fragment 3. The SER to ASN transition introduces a strong steric effect, which may expose the loop L3 in a place potentially unfavorable to interact with p53. Moreover, the L1a and L1b loop areas may be affected, therefore contributing to the loss of affinity of the mutant BRCT to p53.

To shed more light on the molecular mechanisms underlying BC/BOC tumorigenesis triggered by the two BRCA1 mutations, only one of whom (W1837R) has been reported to be as a deleterious/high-risk variant (34), we have performed a clonogenic soft agar growth assay, a technique commonly used to test *in vivo* tumorigenicity (35).

The clonogenic soft agar growth assay performed on HeLa cells stably transfected with either wild-type or mutant *BRCA1* (BRCA1<sup>W1837R</sup> and BRCA1<sup>S1841N</sup>) reveals, in the wt-BRCA1-overexpressing cells, a significant decrease of the clonogenic growth, according with the data of Thompson *et al* (36), while the BRCA1<sup>W1837R</sup>-transfected cells mimic parental HeLa cells. A significant reduction of cell growth in soft agar is found in BRCA1<sup>S1841N</sup>-transfected cells. We believe that the conformational rigidity of mutant BRCA1 induced by the W-R substitution titrates out p53, which is in turn unable to exert its anti-oncogenic role; on the contrary, the S-N aa change might lose the interaction between BRCA1 and p53, making it highly unstable and therefore functionally impaired.

BRCA1 is known to bind to p53 with a bi-partite mechanism, involving both N- and C-terminus. Even though in this study only the C-terminal region of BRCA1 was challenged with p53, nevertheless we believe that the perturbations produced on the folding of the BRCT domain by the two distinct missense mutations are able, *per se*, to functionally impair the interaction between the two partners and the biological pathways regulated by this interaction.

In conclusion, this study provides evidence that the BRCA1<sup>W1837R</sup> and BRCA1<sup>S1841N</sup> variants play a pathogenic role in breast carcinogenesis and strengthen the concept that *in vitro* and *in vivo* assays devoted to test the effects of 'unclassified' BRCA1 mutants are essential for genetic counselling.

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