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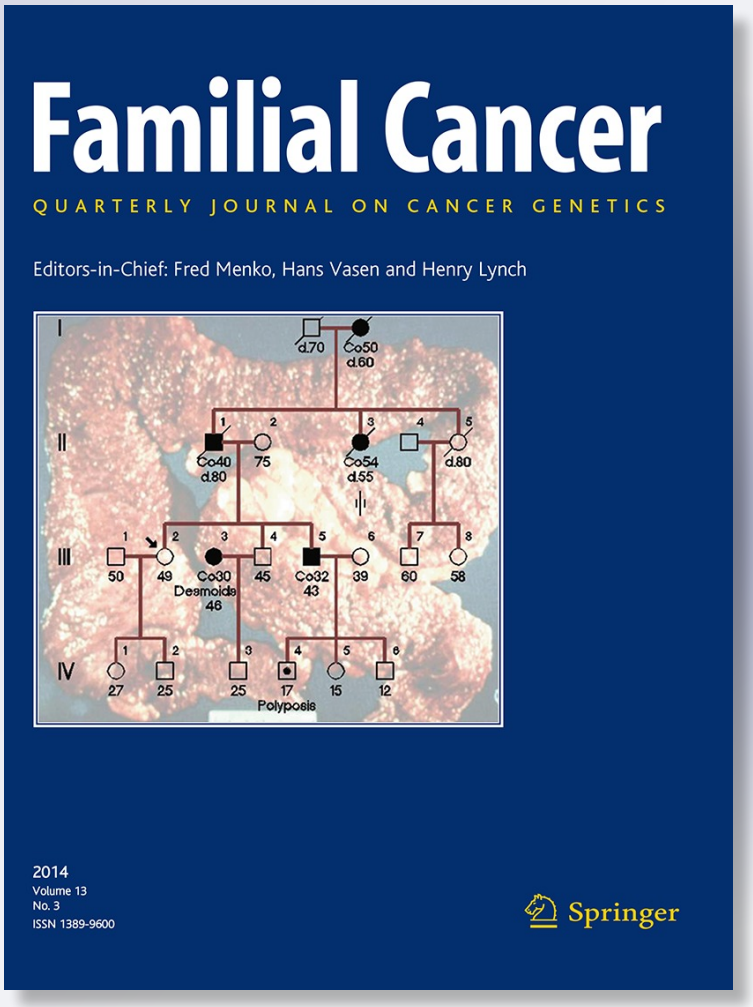

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ORIGINAL ARTICLE

BRCA1 point mutations in premenopausal breast cancer patients from Central Sudan

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Abstract Premenopausal breast cancer (BC) is one of the most common cancers of women in rural Africa and part of the disease load may be related to hereditary predisposition, including mutations in the *BRCA1* gene. However, the *BRCA1* mutations associated with BC in Africa are scarcely characterized. We report here 33 *BRCA1* point mutations, among which 2 novel missense variants, found in 59 Central Sudanese premenopausal BC patients. The high fractions of mutations with intercontinental and uniquely African distribution (17/33, 51.5 % and 14/33, 42.4 %, respectively) are in agreement with the high genetic diversity expected in an African population. Overall 24/33 variants (72.7 %) resulted neutral; 8/33 of unknown significance (24.3 %, including the 2 novel missense mutations); 1 (3.0 %) overtly deleterious. Notably, in silico studies predict that the novel C-terminal missense variant c.5090G>A (p.Cys1697Tyr) affects phosphopeptide recognition by the BRCA1 BRCT1 domain and may have a pathogenic impact. Genetic variation and frequency of unique or rare mutations of uncertain clinical relevance pose significant challenges to *BRCA1* testing in Sudan, as it might happen in other low-resource rural African contexts.

Keywords BRCA1 · Breast cancer · Sudan · Africa · Germline mutations · Premenopausal

Introduction

Mutation analysis of the *BRCA1* gene (MIM#113705) has revealed a wide range of genetic variability, often with an ethno-geographic basis (collated in the three major *BRCA1* databases, i.e., the Leiden Open Variation Database

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(LOVD, http://www.genomed.org/LOVD/), the NIH Breast Cancer Information Core Database (BIC, http://research.nhgri.nih.gov/bic/), and the Universal Mutation Database (UMD, http://www.umd.be/BRCA1/). The functional and pathological consequences of much of this variability are difficult to assess, particularly in case of missense, silent and intronic mutations, that cannot be directly associated with protein inactivation [1–4]. Several biochemical, biophysical, functional and bioinformatics approaches designed to infer the pathological effects of such mutations have been reported [3–5] and computational modeling has also been employed to predict the functional implications of missense mutations [6–9]. The *BRCA1* missense mutations associated with disease are located in the N-terminal RING or C-terminal BRCT domains, suggesting that these regions are critical for the tumor suppressor activity of the protein [3, 10, 11].

The African origin of modern humans accounts for a higher level of genetic diversity in Sub-Saharan Africa, a fact that is important for studies on the susceptibility to complex diseases, including breast cancer (BC) [12]. However, the spectrum of *BRCA1* mutations associated with BC in Africa is still poorly characterized, as few investigations have been thus far conducted [13–15]. Furthermore, the clinical interpretation of the uniquely African *BRCA1* variants is often confounded by the paucity of information on personal (other than BC) and family history of cancer, an inevitable consequence of the low accessibility to health care and of the lack of awareness of cancer in rural Africa [15–17].

In Sudan BC is the most frequent hospital-treated malignancy, characterized by occurrence in multiparous premenopausal women, advanced stage at diagnosis, high grade and triple negative phenotype, characteristics akin to those of *BRCA1*-associated hereditary BC [18, 19].

We previously described the *BRCA1* and *BRCA2* mutations detected in a series of Central Sudanese BC patients within 40 years of age or of male gender, consecutively diagnosed between 2001 and 2002 [20]. That study provided evidence of a diverse mutational spectrum with several unique variants, and suggested that *BRCA1* and *BRCA2* importantly contribute to BC in Sudan [20]. Here we report the results of *BRCA1* mutation analysis on a distinct group of 59 premenopausal Sudanese BC patients not included in our previous study [20].

Patients and methods

Patients

This study was approved by the Ethical Committees at the National Cancer Institute, University of Gezira (NCI-UG) and "*G. d'Annunzio*" University. Fifty-nine premenopausal BC patients (age at diagnosis $\leq$45 years) for whom whole blood had been sampled were selected among the BC cases treated at the NCI-UG from November 1999 through December 2004. None of the selected cases had been analyzed in our previous study [20]. An informal consent, according to guidelines set by the NCI-UG, had been obtained from each patient at blood sampling. The mean age at BC diagnosis was 37.4 $\pm$ 6.2 years (range 23–45 years), the mean age at menarche 13.9 $\pm$ 1.2 years; range 12–17 years). The patients were mostly home-resident women (46/59, 78 %) from rural villages in the area of the Gezira Irrigation Scheme. Only one patient (1.7 %) reported a family history suggestive of cancer, without further specifications, and one (1.7 %) was diagnosed with bilateral BC.

Genetic testing

Genomic DNA was isolated from blood using either the QIAamp DNA Blood Mini Kit (QIAGEN Inc, Chatsworth, CA) or the silica-based method [21]. The entire *BRCA1* coding sequence, including intron–exon boundaries, was analyzed by DHPLC using the Wave®Nucleic Acid Fragment Analysis System (Transgenomic Inc. San Jose, CA). Primers for *BRCA1* exons 2–24 and PCR and DHPLC conditions were as reported in the literature [22]. The amplicons with altered DHPLC profiles were sequenced using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA) or the MegaBACE™ 1000 DNA Analysis System (Amersham GE Healthcare Biosciences, Cologno Monzese, Italy). Variants nomenclature follows the guidelines of the Human Genome Variation Society (HGVS). All detected variants were confirmed on replicate DNA aliquots. Large deletions/rearrangements (LRs) could not be assessed, because the patients, all with advanced disease, had been sampled several years before genetic analyses and, for most, only limited amounts of DNA were available. Anyhow, *BRCA1* LRs rates seem to be relatively low in BC patients of African origin [23, 24].

The clinical significance of the identified variants was assessed based on information obtained from three major *BRCA1* databases, LOVD, BIC and UMD. Variants of unknown significance are referred to as VUS [25, 26]. The geographic distribution of the previously reported variants was assessed based on the three above-referred databases plus the International HapMap project (HapMap3, http://hapmap.ncbi.nlm.nih.gov/index.html.en) [27], the 1000 Genomes Project (http://www.1000genomes.org/) or relevant literature not included in the databases. Variants having minor allele frequencies (MAFs) >1 % reported in the above mentioned databases or in the literature, were considered neutral [27], those detected in unique patients

(as well as deleterious mutations) were analyzed in a control population of 180 individuals (111 males and 69 females) from the Wad Medani city region (mean age 36.1 ± 10.4 years, range 20–70 years; male/female ratio 111/69; mean age of females 38.4 ± 10.1 years, range 20–65 years; mean age of males 34.7 ± 10.4 years; range 20–70 years). These variants were regarded as VUS when MAFs resulted ≤1 % considering the entire Sudanese population sample tested (i.e., 59 patients and 180 controls).

In silico studies

Bioinformatics analyses of the unique missense variants with novel or VUS status were performed using Align-GVGD (http://agvgd.iarc.fr/) [6, 28], PolyPhen version-2 (PolyPhen-2) (http://genetics.bwh.harvard.edu/pph/) [29, 30] and SIFT (release 3.0), (http://sift.bii.a-star.edu.sg/) [31]. The Human Splicing Finder version 2.4 (www.umd.be/HSF) was used to predict effects on potentially-affected mRNA splice sites [32]. This tool uses different algorithms (Human Splicing Finder, ESE Finder, ESE Rescue and MaxEnt) to calculate consensus values [4]. For the novel structural variant p.C1697Y molecular modeling and 3D structure computer analysis were performed starting from the X-ray coordinates of the human BRCA1-BRCT domain [33], downloaded from the Protein Data Bank (www.rcsb.org; PDB accession code: 1t15) [34]. The model was constructed by residue replacement and energy optimization in the X-ray structure using MODELLER 9.12 [35]. The mutation-dependent conformational change was predicted using the ModLoop web server (http://salilab.org/modloop), which applies the satisfaction of spatial restraints approach of the Modeller loop modeling routine [36]. Mutant stability was evaluated using the Fold-X empirical force field [37], that calculates the free energy change in the protein upon point mutation. Images were obtained with PyMol (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC).

Results and discussion

A total of 33 *BRCA1* variants were detected in 49/59 BC patients (Table 1). These comprised 2 novel variants (i.e., *c.1088A>G*: p.Asn363Ser and c.5090G>A: p.Cys1697Tyr), and 31 variants previously reported in the BIC, and/or UMD, LOVD, HapMap3 and 1000 Genomes databases or in the literature [20, 27, 38–40]. The occurrence of the previously reported variants in world populations was assessed from the databases or relevant literature: 17/33 variants (51.5 %) were found in populations from at least three continents, and 14/33 (42.4 %, including the 2 novel variants) only in Africans and/or individuals of African ancestry (Table 1). The high fractions of mutations with intercontinental and uniquely African distribution are consistent with the high genetic diversity expected in an African population [12].

The clinical interpretation of the variants is complicated by the lack of information on cancer family history, that reflects lack of awareness of neoplasms in the traditional Sudanese culture [16]. The detected variants were classified relying on data reported in the LOVD, BIC and UMD databases, on MAFs from HapMap3 [27], the 1000 Genomes Project and/or the screened Sudanese subjects (59 patients and 180 controls). Overall 24/33 variants (72.7 %) resulted neutral (N); 8/33 of unknown significance (VUS) (24.3 %, including the 2 novel mutations); 1 (3.0 %) overtly deleterious (D) (Table 1).

The deleterious mutation, c.4986+6T>C, located in the exon–intron boundary of exon 16 and found in the youngest of our patients (25 years-old), was previously described in an American Caucasian [40]. This mutation introduces an aberrant cryptic splice site and an in-frame stop codon at position 1662. Of the 8 VUS, 6 were located in the coding sequence (Table 1). These included the 2 novel mutations (c.1088A>G, p.Asn363Ser; c.5090G>A, p.Cys1697Tyr), and 4 rare variants already in LOVD, BIC and UMD for Africans and African-Americans, i.e., c.503A>C, (p.Lys168Thr); c.1846_1848delTCT, Del Ser cod.616, reported in trans with a pathogenic mutation [41]; c.2109A>G, p.Thr703Thr and c.2773A>C, p.Ile925Leu. Two of these, c.1088A>G, p.Asn363Ser and c.2773A>C, p.Ile925Leu, were classified as VUS based on MAFs ≤1 % but are predicted to be neutral based on bioinformatics analyses in Table 2. In addition, there were two VUS in non-coding regions, i.e., c.301+55G>A, described in Africans, and c.5074+108G>A, described in Europe (UMD) (Table 1). Thus 7 of 8 VUS were either novel or previously identified in individuals of African origin. None of these VUS was detected in the Sudanese controls (Table 1). Interestingly, Lys168Thr was associated with 3 known variants, Tyr179Cys, Phe486Leu, and Asn550His (Table 1), which occur together and may represent a rare haplotype of no pathologic relevance [42].

Bioinformatics analysis was performed to evaluate possible functional implications of the eight VUS [43]. Prediction of the effects on mRNA splicing, based on Human Splicing Finder version 2.4 (http://www.umd.be/HSF) [32], suggested functional consequences only for c.503A>C (p.Lys168Thr) (Table 2). Prediction of amino acid substitutions with SIFT, PolyPhen and Align-GVGD (Table 2) concordantly supported a deleterious effect only for the novel VUS c.5090G>A (p.Cys1697Tyr) [Align-GVGD category: C65; prior probability: 81 (95 % CI 061–0.95)] [25, 26].

Table 1 Classification and occurrence in world populations of the 33 *BRCA1* variants identified in 49 out of the 59 Central Sudanese premenopausal BC patients studied

Nucleotide change(s)	Protein change	SNP	Clinical significance[a]	Sudanese patients	Occurrence in world populations[a]
In-frame deletion					
c.1846_1848delTCT	Del Ser cod.616	–	VUS	1	A, A–A
Missense					
c.503A>C[b]	p.Lys168Thr	–	VUS	1[e]	A
c.536A>G	p.Tyr179Cys	rs56187033	N	1[d,e]	E, A–C, A–A
c.557C>A	p.Ser186Tyr	rs55688530[c]	N	2	A [20], A–A
c.1088A>G	**p.Asn363Ser**	**–**	**VUS**	**1**	**Sudan**
c.1456T>C	p.Phe486Leu	rs55906931[c]	N	1[e]	Global [38]
c.1648A>C	p.Asn550His	rs56012641[c]	N	1[e]	Global [38]
c.2077G>A	p.Asp693Asn	rs4986850	N	1	Global
c.2167A>G	p.Asn723Asp	rs4986845	N	1	E, A–A
c.2612C>T	p.Pro871Leu	rs799917	N	25	Global
c.2773A>C	p.Ile925Leu	rs4986847[c]	VUS	1	A, A–A
c.3113A>G	p.Glu1038Gly	rs16941	N	19	Global
c.3548A>G	p.Lys1183Arg	rs16942	N	23	Global
c.4837A>G	p.Ser1613Gly	rs1799966	N	21	Global
c.5090G>A	**p.Cys1697Tyr**	**–**	**VUS**	**1**	**Sudan**
Synonymous					
c.2082C>T	p.Ser694Ser	rs1799949	N	9	Global
c.2109A>G	p.Thr703Thr	rs4986844[c]	VUS	1	A, A–A
c.2311T>C	p.Leu771Leu	rs16940	N	21	Global
c.4308C>T	p.Ser1436Ser	rs1060915	N	14	Global
Intronic					
c.1-134T>C	–	rs3765640	N	5	Global
c.301+55G>A		rs182724216[c]	VUS	1	A [20]
c.442−34C>T	–	rs799923	N	2	Global [20]
c.548−58delT	–	rs1799736	N	11	Global [20]
c.4485−63C>G	–	rs8176212	N	18	Global
c.4986+6T>C	Splice	rs80358086	D	1	A–C
c.4987-68G>A	–	rs81766234	N	21	Global
c.4987−92A>G	–	rs8176233	N	21	Global
c.5074+65G>A	–	rs8176235	N	7	A, A–A
c.5074+108G>A	–	–	VUS	1	E
c.5152+66G>A	–	rs3092994	N	21	Global
c.5406+8T>C	–	rs55946644	N	4	A–A, A–C, A [20]
c.5468−10C>A	–	rs8176316	N	1	A–A, A [20]
c.36C>G[e]	–	rs3092995	N	5	A [20]

Novel mutations are in bold

D deleterious, *N* most likely neutral, *VUS* variant of unknown clinical significance, *E* European, *A-A* African-American, *A-C* American-Caucasian, *A* African

[a] Data based on the BIC, UMD, LOVD, HapMap and 1000 Genomes databases and on 2 references not reported in these databases [20, 38]

[b] Reported as VUS in BIC, this mutation was detected in a female patient of mixed ancestry (Cape Colored)

[c] Minor allele frequency <1 % in the 1000genomes and/or HapMap3 databases

[d] Also detected in the control Sudanese population (360 chromosomes). Global: variant found in populations from at least 3 continents

[e] These four variants were detected in a unique patient, and three of them (Tyr179Cys, p.Phe486Leu, p.Asn550His) were previously reported on the same putatively not pathogenic haplotype [42]

Table 2 Bioinformatics analyses of VUS variants for protein changes and mRNA splicing

Variant (Protein change)	Prediction aminoacidic change			Prediction splice signal difference			
	SIFT	PolyPhen	(a) Align-GVGD	ESE-finder	(b) HSF (variation)	RESCUE-ESE	(c) MaxEnt Branch Point
c.503A>C () (*p.Lys168Thr*)	NT	PoD ++	C0	1 × SB	1 × SB (−42.5 %)	3xSB	3′ NS (+71.07 %)
c.1088A>G) (*p.Asn363Ser*)	**T**	**B**	**C0**	**No**	**1 × NS (+72.74 %)**	**1 × SB**	**No**
c.1846_1848delTCT (*Del Ser cod.616*)	na	na	na	1 × SB	1 × NS (+84.82 %) 1 × SB (−82.21 %)	No	No
c.2109A>G (*p.Thr703Thr*)	na	na	na	No	No	2 × NS	5′ NS (+302.38 %)
c.2773A>C (*p.Ile925Leu*)	NT	B	C0	1 × SB 1 × NS	No	No	5′ SB (−31.20 %) 3′ NS (+31.62 %)
c.5090G>A (p.Cys1697Tyr)	**NT**	**PrD +++**	**C65**	**1 × SB**	**2 × SB (−16.79 %; −40.17 %)**	**1 × NS**	**No**
c.301+55G>A	na	na	na	1xSB	No	1 × SB	5′ SB (−36.24 %) 3′ SB (−41.7 %)
c.5074+108G>A	na	na	na	No	1 × New Site (+59.5 %)	No	5′ NS (+186.71 %)

Novel variants in bold

(a) Align GVGD categories range from C0, least likely to interfere with function, to C65, most likely to interfere with function

(b) Percent differences between wild-type and variant <5 % were excluded

(c) Percent differences between wild-type and variant <25 % were excluded

na not applicable for synonymous or intronic change, *HSF* Human Splicing Finder, *No* no variation between mutant and reference sequence found with this matrix, *T* tolerated, *NT* not tolerated, *PoD* possibly deleterious, *PrD* probably deleterious, *B* benign. 1/2/3xSB, 1/2/3 site(s) broken, *NS* new site created

The novel missense mutation C1697Y is located in the C-terminal tandem repeat domain (BRCT1) of *BRCA1*. This region is involved in binding of important BRCA1 interactors, such as corepressor CtIP and the helicase BACH1 [44, 45] and possesses a transcription-activation function [46, 47]. A large portion of the *BRCA1* mutations linked to breast and ovarian cancer is located in the BRCT motif, implying a link between BRCT function and the BRCA1-mediated tumor suppression [3, 10].

Visual inspection of the X-ray-solved 3D structure of BRCT in complex with the BACH1 peptide [25] showed that this domain includes two structurally similar BRCT repeats, packed together with a head-to-tail topology and connected by a 23 residues flexible linker. The BRCT–BACH1 interaction is mediated by a hydrophobic surface involving the loop 1689–1699, that includes cysteine 1697 (Fig. 1). Cysteine 1697 does not directly participate in phosphopeptide binding, however residues 1689–1699 are precisely arranged to assume a conformation that accommodates the positively-charged phosphorylated serine (+3) of the BACH1 ligand. Cysteine 1697 is adjacent to arginine 1699, essential to establish two stabilizing H-bonds with BACH1 [48]. Modeling of the Cys1697Tyr mutant by residue replacement showed that the bulky side chain of the tyrosine did not fit into the packed conformation of this region and was therefore flipped outwards. Ab-initio modeling of the whole loop predicted a large conformational change, which most likely altered the phosphopeptide interaction surface. A probable deleterious effect of the mutation was highlighted by different tools that predicted a destabilizing effect, including Polyphen-2, I-mutant (http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant2.0/I-Mutant2.0.cgi), Auto-mute [49] and Cupsat [50]. Mutant stability was also evaluated with the FoldX force field [37], that calculates free energy change ($\Delta\Delta G$) upon mutation. The $\Delta\Delta G$ corresponded to 13.57 kcal/mol, indicating a highly destabilizing effect, with the strongest component corresponding to van der Waals clashes (17.71 kcal/mol). This suggests that the perturbing effect of the mutation is mainly due to the steric hindrance of the tyrosine side chain. Structural alignment of the BRCA1 BRCT domain with characterized BRCT domains in other proteins show that the loop regions corresponding to the BRCA1 residues 1689–1699 differ in length and conformation (Fig. 2). The loop has a reduced length and a flat conformation when the position corresponding to BRCA1 Cys1697 is occupied by bulky residues (isoleucine, histidine, and phenylalanine). The fold assumed in the BRCA1 BRCT is typically

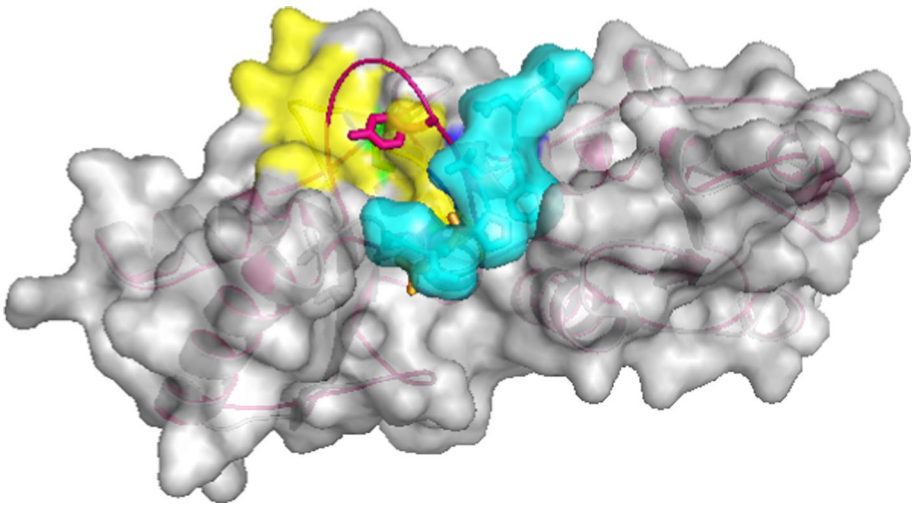

Fig. 1 Molecular surface representation of the BRCA1 C-terminal BRCT tandem repeat in complex with the BACH1 helicase phosphopeptide (pdb code 1t15). The *yellow* surface corresponds to loop 1689–1699, the *green* and *blue* spots match with the cys1697 and arg1699 positions, respectively. BACH1 is in *cyan*, with the phosphoserine in *orange*. The model of the mutant C1687Y is superposed, with loop 1689–1699 and tyrosine 1697 in *pink*. (Color figure online)

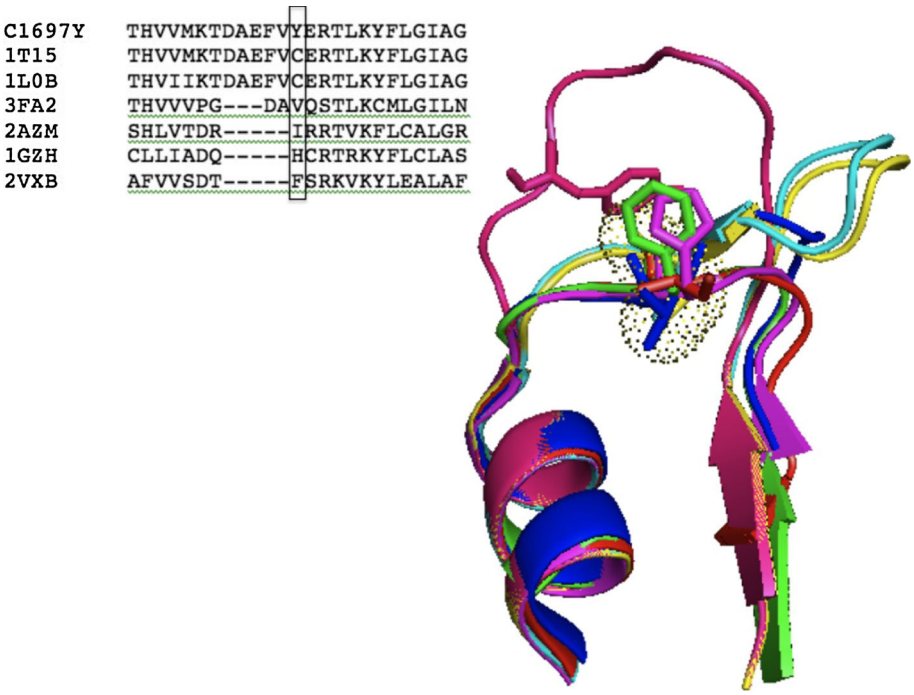

Fig. 2 Multiple sequence and 3D structural alignments of the loop portions of the mutant C1697Y (*pink*) and of the human and rat BRCA1-BRCT domains (PDB: 1t15, *yellow* and 1l0b, *cyan*), with the homologous domains of the human BARD1 (3fa2, *blue*), MDC1 (2azm, *red*), 53BP1 (1gzh, *magenta*) and CRB2-BRCT2 (2vxb, *green*) proteins. The position corresponding to cys1697 (*dots*) can be occupied by different residues, such as isoleucine, valine, histidine and phenylalanine. The two cysteine-carrying loops (*yellow* and *cyan*) are longer than the other loops and assume a hairpin shape. In the mutant, a large shape modification is predicted to accommodate the bulky tyrosine in the loop. (Color figure online)

observed with a cysteine side chain in position 1697. In the C1697Y mutant, a large shape modification would be required to accommodate the bulky tyrosine in the loop. The consensus results of the in silico analyses highlighted the adverse effect of a tyrosine side chain in position 1697 and supported the hypothesis of functional impairment.

A germline variant of Cysteine 1697 to Arginine has already been identified in Scandinavian breast and ovarian cancer families [33] and Vallon-Christersson et al. [51] showed that the BRCA1 construct containing the mutation C1697R was not able to activate transcription in the yeast (*S. cerevisiae*) two-hybrid system, supporting C1697R as a pathogenic mutation. Furthermore, the structural determinants of phosphopeptide recognition by BRCA1-tandem BRCT domains were investigated by kinetic and thermodynamic approach [52]. The study demonstrated that two equilibrium conformations are represented in the interaction mechanism and speculated that the observed flexibility could facilitate the binding of BRCA1 to different phosphorylated interactors [52]. This observation may help to interpret the effect of pathological mutations that are not directly involved in peptide interaction, as responsible for an alteration of the equilibrium between the two conformations. Here we suggest that the structural effects of the C1697Y mutation may be related to a distortion or unfolding of the 1689–1699 loop conformation,

which can be propagated to functionally crucial portions of the protein, such as the peptide recognition surface. An in-depth evaluation of this hypothesis would require simulations with more sophisticated techniques, such as molecular dynamics, together with experimental functional tests of the mutant protein.

In conclusion, the present study adds to the results of our previous report [20], confirming that Central Sudanese premenopausal BC patients show a high number of rare *BRCA1* variants, a finding in agreement with the deep evolutionary history of humans in Africa and with former evidence that high variation in *BRCA1* is found in African BC patients [12, 15, 20, 27]. We also confirm that pathogenic *BRCA1* mutations occur in unselected Sudanese premenopausal BC patients and report at least one new variant, in the BRCT domain, most likely responsible for BC. Overall this study, as other investigations conducted on African populations, contributes to a better understanding of the global genetic variation of *BRCA1*. High genetic variation, frequency of unique mutations of uncertain functional relevance, costs of screening, and lack of reliable cancer family history data and, hence, difficult applicability of results to BC prevention, might pose significant challenges to clinical *BRCA1* genetic testing in Sudan, as in other low-resource rural African contexts [16, 17].

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Conflict of interest The authors declare that they have no conflict of interest.

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