RESEARCH ARTICLE

REVISED *BRCA1* novel variation V1736D and *in silico* analysis of SNP Q356R in Sudanese patients with breast cancer [version 4; peer review: 1 approved, 2 not approved]

Previously titled: *BRCA1* novel mutation V1736D and *in silico* analysis of SNP Q356R in Sudanese patients with breast cancer

Mohamed Elmogtba Mouaweia Mohamed Aabdein^{1,2},
 Alsmawal Awad Mohammed Elimam ¹⁻³, Hisham N. Altayb^{4,5},
 Mohamed El-Fatih Mohy Eldeen³⁺, Mosab Mohamed Gasemelseed^{4,6},
 Afra AbdElhamid FadlAlla⁴, Marwa Mohamed Osman⁴, Soada Ahmed Osman⁴,
 Hajir Ali Saeed⁴, Mona ShamsAldeen Ali^{4,7}, Tomador Siddig^{4,7},
 Reem Abdelrahman Osman⁴, Rehab Ahmed Elhadi^{4,7},
 Muzamil Mahdi Abdel Hamid ⁸, Mohamed Ahmed Salih⁴

¹Department of Histopathology and Cytology, Postgraduate College, Al-Neelain University, Khartoum, Sudan

²Daoud Research Group, Khartoum, Sudan

³Department of Histopathology and Cytology, Al-Neelain University, Khartoum, Sudan

⁴Department of Biotechnology, Africa City of Technology, Khartoum, Sudan

⁵Department of Microbiology, Faculty of Medical Laboratory Sciences, Sudan University of Science and Technology, Khartoum, Sudan

⁶Faculty of Medical Laboratory Sciences, Al-Neelain University, Khartoum, Sudan

⁷Department of Laboratories and Blood Banking, Omdurman Teaching Hospital, Omdurman, Sudan

⁸Institute of Endemic Diseases, University of Khartoum, Khartoum, Sudan

+ Deceased author

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Abstract

Background: Breast cancer (BC) remains one of the leading causes of death in women worldwide. The *BRCA1* deleterious mutation has a significant role in developing BC, and the risk has been estimated to be 46–87%. Many studies emphasize the need for mining *BRCA1* gene mutations that might have a role in BC pathogenesis and could affect early disease onset. This study was conducted to screen for possible pathogenic single nucleotide polymorphisms (SNPs) in *BRCA1*,

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targeting three regions: two in exon 11 and the third in exon 20.

Methods: 45 blood samples were collected from patients diagnosed with BC. DNA was extracted and selected regions were amplified by PCR using three sets of primers - two within exon 11 and one within exon 20 of *BRCA1*. Subsets of 10 samples were selected for each primer set (30 PCR products) and sequenced. Sequences were analyzed using various bioinformatics tools.

Results: Two missense variations were found, Q356R (rs1799950) in one patient (27 years old) and a novel SNP, V1736D, in three premenopausal patients (≤ 45 years), which were located within exons 11 and 20, respectively. Both detected variants were heterozygous, a status found in all patients detected with such monoallelic variation. Both missense variants underwent *in silico* analysis. The well-known variation, rs1799950, was predicted to alter the protein activity, conferred by a mutant residue (R-Arg), owing to the position with a bigger size and positive charge. The novel SNP, V1736D, was predicted to play a role in the pathogenesis of BC.

Conclusion: Both variants require further investigation, firstly to assess their contribution to BC and secondly to determine their potential diagnostic value when assessed in a larger population.

Keywords

breast cancer, BRCA1 gene, SNP, novel mutation, missense variants, heterozygous, Sudanese patients

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- Habiba Chaabouni-Bouhamed**, University of Tunis El Manar, Tunis, Tunisia
Centre Urbain Nord, Tunis, Tunisia
- Mahmoud Balbaa** , Alexandria University, Alexandria, Egypt
- Khalid Dafaallah Awadelkarim**, University of Gezira, Wad Madani, Sudan

Any reports and responses or comments on the article can be found at the end of the article.

Corresponding authors: Mohamed Elmogtba Mouaweia Mohamed Aabdein (mogtaba8788@hotmail.com), Alsmawal Awad Mohammed Elimam (toteil1@hotmail.com)

Author roles: Aabdein MEMM: Conceptualization, Data Curation, Methodology, Resources; Elimam AAM: Conceptualization, Data Curation, Investigation, Methodology, Resources, Software, Writing – Original Draft Preparation; Altayb HN: Formal Analysis, Investigation, Software, Validation, Visualization; Eldeen MEFM: Data Curation, Formal Analysis, Methodology, Validation, Visualization; Gasemelseed MM: Investigation, Software, Writing – Original Draft Preparation; Fadlalla AA: Investigation, Software, Writing – Original Draft Preparation; Osman MM: Investigation, Software, Writing – Original Draft Preparation; Osman SA: Investigation, Software, Writing – Original Draft Preparation; Saeed HA: Investigation, Software; Ali MS: Investigation, Software, Writing – Original Draft Preparation; Siddig T: Investigation, Software, Writing – Original Draft Preparation; Osman RA: Investigation, Software; Elhadi RA: Investigation, Software; Hamid MMA: Data Curation, Formal Analysis, Methodology, Validation, Visualization, Writing – Review & Editing; Salih MA: Conceptualization, Formal Analysis, Investigation, Project Administration, Software, Supervision, Validation, Visualization, Writing – Review & Editing

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REVISED Amendments from Version 3

In response to (Referee 3) Dr Khalid Dafaallah Awadelkarim:

- The title of our article has been edited to further describe the nature of the findings; "variation" is more suited than "mutation" in terms of practice.
- Introduction – the fourth paragraph: the *BRCA2* gene has been added as part of the screened investigations of the two studies. Variants information of the second study has been expanded.
- Methods – "Sampling" subsection: the mean age standard deviation has been expressed.
- Results – "Bioinformatics assessment" subsection: we highlight the HGVS way of protein nomenclature expression
- Discussion – we named both domains and expanded on the novel variant mentioned in the Biunno *et al.*, 2014 study
- **Table 3** was added to highlight patient sequencing results after sequence validation assessments, and cited in the Methods and Discussion.

See referee reports

Introduction

Breast cancer (BC) is a very serious issue worldwide, and is one of the leading causes of death in women today. In the US, it was estimated that there were approximately 232,670 new cases of BC and 40,000 BC deaths in 2014, and the number increased with 13,990 new cases and 450 deaths in 2016^{1,2}. In Africa, in 2012, the rate was about 94,000 women with BC, which resulted in 48,000 deaths³, and studies in Africa have described a poor outcome with a late diagnosis, due to the aggressiveness of the disease and the absence of screening programs^{4–7}.

In Sudan, BC occurs at the highest frequency among women compared to other types of cancer^{8–13}. In a hospital-based statistical report in Sudan¹³, BC was found to be the most commonly diagnosed malignant tumor and was characterized by early onset and bad prognosis. The report showed invasive ductal carcinoma to be the predominant type (82%), and 74% of patients were <50 years old with an advanced disease stage, indicating that most cases remain undiagnosed for long periods^{8,9,13–15}. *BRCA1* (OMIM_113705) was mapped in 1994 and subsequently cloned. It is located on chromosome 17 region 2, band 1 (17q21), which is responsible for encoding 1863 amino acids¹⁶. Since 1995, the *BRCA1* tumor suppressor protein has been found to arrest cell proliferation, play an important role in the repairing process of DNA damage, and was suggested to have a role in cell cycle regulation through interacting directly or indirectly with other regulatory molecules^{17–22}. *BRCA1* when it is altered becomes deficient, and such loss of function mutates the protein, which not only perturbs chromosomal integrity and genome stability, but increases the mutation rate of other genes^{23–26}. Therefore, it has been proposed that *BRCA1* doesn't directly initiate cancer formation, but enhances the process by making the affected cells highly susceptible to malignant transformation^{27,28}. Germline mutations in *BRCA1* are responsible for a large proportion of inherited predispositions for BC, and individuals that carry an inherited mutation in the *BRCA1* gene have a significant risk (46–87%) for

developing BC by 70 years of age^{29–34}. Regionally among Africa and locally in Sudan, *BRCA1*-associated BC has been identified among premenopausal women^{6,9,35}. This indicates the highly susceptible nature of such a mutation to enhance cancer development earlier during fertile reproductive women. African scientific literature has greatly studied the risk of BC resulting from reproductive factors (such as early menarche, late menopause, and sex hormones), but has not explored much into the genetic predisposition of the disease^{6,36–38}.

About 13108 SNPs have been reported within human *BRCA1*, of them 1608 were reported as missense variants and about 151 have been identified to be pathogenic, according to the SNP database at NCBI (<http://www.ncbi.nlm.nih.gov/snp>). Most germline mutations result from small insertions/deletions frameshift, stop codon or deletion, duplication at exon/intron sequence levels which are met by disruption of the mutated splice sites, altogether resulting in a nonfunctional truncated protein³⁹. The SNP variant rs1799950 was observed to have a **negative association with BC, by favoring** more frequently the control groups than BC patients^{40,41}. This SNP was tested among familial *BRCA1* carriers for BC risk association, and a significant association was found within affected families⁴². When the SNP was haplotype-homozygous within affected families, the risk was increased, as in the case of sporadic risk association study⁴³. The heterozygosity nature of this mutation has been noticed within different studies, and some studies found the heterozygous variant more frequently within controls, hence it was adversely associated with BC, while in another studies, the homozygous variant was found more frequently within BC patients^{40,43,44}. In addition, heterozygosity was functionally assessed among monoallelic *BRCA1* mutation carriers of rs1799950, and the results showed that such an alteration could permit variation in protein expression and activity in a haploinsufficient way, which could alter the cell's normal behavior and result in tumor transformation by enhancing tumorigenesis. Such variation conferred by one mutated copy suggests that the wild-type copy alone is not capable of compensating the loss of the other wild allele^{45,46}. Turkovic *et al.* found that such a haplotype association was noticed more frequently within deleterious mutation carriers; however, this was observed in a small sample size⁴⁷. In addition, some studies have found rs1799950 to be associated with early-onset prostate cancer^{48–50}.

Two genetic studies have been conducted in Sudan concerning *BRCA1* and *BRCA2*. One was a survey of 2370 students at a girl's secondary school in Northern Sudan-Marawi, in which the study divided 67 students into two groups (47 students with a family history of BC and 20 with unaffected families) to analyze *BRCA1* and *BRCA2* mutations. In the first group, which was 2.37% of responders, the frequency of mutations was higher for *BRCA1*, and most mutations were within exon 11. The study continued to recommend further assessments of this region in subsequent local projects, and this formed the basis of our primer (1 and 2) selection within the present study¹⁰. The other study, from central Sudan, investigated 34 early onset premenopausal women patients diagnosed with BC (<40 years) and one male patient. The study identified 60 variations in these patients, five of which

were deleterious, affecting the outcome protein; two out of them were identified within *BRCA1* exon 11 (c.3999delT and c.4065_4068delTCAA) in one patient each. The other 55 were named non-truncating, five of them were predicted as pathogenic, and these included four variants (3 missense and a deletion at splice acceptor site - intron 2) which were previously found as unclassified variants UV in addition to a novel change. Two of the unclassified missense variants were identified within *BRCA1* exons 9 and 11 (c.557C>A and c.2458A>G), the first was found more frequent (4 patients)⁹.

From the same region within central Sudan, early onset BC premenopausal women have also been investigated for *BRCA1* point mutations. The findings revealed the presence of one deleterious variant, 24 neutral variants and eight variants of unknown significance, within which two novel variants were discovered³⁵.

Since there have been scarce genetic studies conducted highlighting genetic characteristic and familial risk status of BC patients in Africa³⁸ our aim was to screen for the type and spectrum of germline mutations in *BRCA1* by focusing on three regions within the gene, two within exon 11 and one within exon 20, using sequencing, and to further assess the detected variants using *in silico* analysis tools. These regions and their selections were based on the quality of available primers (e.g. best GC content, adequate length, according to previous literature⁵¹), previous local research findings revealing frequent mutations within exon 11^{9,10}, and the cost.

Methods

Sampling

This study was carried out in March 2015 at the Radiation and Isotope Center in Khartoum. 2–3 mls of blood were collected randomly from 45 patients diagnosed with BC who attended the center for treatment and follow-up (no other inclusion/exclusion criteria were relevant), using sterile EDTA-K3 vacutainer and kept at -20°C. All the patients were aged between 27 and 80 years old, with a mean of 45.9 ± 12.5 years. Early onset cases were more frequent than late onset: 25 (55.6%) cases with early onset, with a mean of 36.6 ± 4.9 years; and 20 (44.4%) cases of late onset with a mean of 57.4 ± 9.0 years. Multiparity was high in 30/45 (66.6%). Six cases reported a family history of BC (13.3%), abortion was detected in 10 cases (22.2%). Of the presented histotypes that were available to us (16 cases unknown), ductal type tumor was more frequent and shown in 22 cases (48.8%), then lobular in five cases and mucinous in two cases. In addition, five cases showed distal cancer metastasis; lung and bone were frequent secondary sites. Right side tumor originated in 20 cases (44.4%), while the left side tumor was found in 15 cases (33.3%), and four cases presented with bilateral BC (6 cases unknown) (see Table 1).

Ethical statement

This study was conducted under the guidelines and approval of the Research Ethics Committee of Sudan Ministry of Health – Khartoum state. All participants provided oral informed consent to participate in the study. Oral informed consent was obtained as opposed to written consent, due to the literacy levels of the

Table 1. Patient demographics and characteristics.

*Comprising both Khartoum 16 cases and AlGezirah 5 cases.

Variable		Frequency, n (%) (n=45)
Onset	Early (≤ 45 years)	25 (55.6)
	Late (≥ 46 years)	20 (44.4)
Family history	Breast cancer	6 (13.3)
	Other cancer	5 (11.1)
	No family history of any cancer	34 (75.6)
Parturition	Multiparous	30 (66.7)
	Nulliparous	13 (28.9)
	Primiparous	2 (4.4)
History of abortion	Yes	10 (22.2)
	No	35 (77.8)
Marital status	Currently married	41 (91.1)
	Single	3 (6.7)
	Previously married	1 (2.2)
Tribe	Ja'alya	5 (11.1)
	Shaygeya	5 (11.1)
	Dnagla	4 (8.9)
	Noba	3 (6.7)
	Rezaigat	3 (6.7)
	Other	25 (55.5)
Geographical region	Central Sudan*	21 (46.7)
	Western Sudan	15 (33.3)
	Northern Sudan	6 (13.3)
	Eastern Sudan	3 (6.7)
Tumor site	Unilateral	35 (77.8)
	Bilateral	4 (8.9)
	Unknown	6 (13.3)

patients and the time limited interaction between researchers and patients at the hospital.

DNA extraction and PCR amplicons

DNA was extracted using the salting-out method⁵² for 45 patients samples. In addition, proteinase K was used to enhance WBC membrane breakdown at 56°C for 1 hour. For PCR, three previously published pairs of primers⁵¹ were used to amplify three regions within the *BRCA1* gene. All the three primers were selected for their quality performance, optimal size and GC content, after being assessed with Oligoanalyzer tool 3.1 (<https://www.idtdna.com/calc/alyzer>). These primers were synthesized by Macrogen Incorporation (Seoul, South Korea; Table 2). Annealing temperatures were adjusted on several runs (Table 2). Maxime PCR PreMix Kit i-Taq 20 μ l (INTRON Biotechnology, South Korea) was used for PCR - 15 μ l distilled water, 3 μ l sample DNA (30 ng/ μ l; as checked by NanoDrop 1000) and 1 μ l of the final concentration of each primer (10 pmol/ μ l forward and reverse). PCR mixture was subjected to initial denaturation step at 96°C for 5 minutes; followed by 35 cycles of denaturation at 96°C for 30 seconds, primer annealing at 50 or 55°C depending on the set used, for 30 seconds; followed by a step of elongation at 72°C for 60 seconds; the final elonga-

Table 2. *BRCA1* sets of primers used for PCR. F: forward; R: reverse; bp: base pair; CDS: coding sequence; V1: variant one.

PRIMERS 5'→3'	EXON	ANNEALING Temp. (°C)	PRIMER SIZE (bp)	PRODUCT SIZE (bp)	PRODUCT REGION
Primer 1 F: 5'CCA AGG TGT ATG AAG TAT GT'3 R: 5'GAT CAG CAT TCA GAT CTA CC'3	11 11	50 50	20 20	442 (NG_005905: 81189bp)	Only 367bp CDS (V1 NM_007294.3)
Primer 2 F: 5'CTC ACT AAA GAC AGA ATG'3 R: 5'CTT TCT GAA TGC TGC TAT'3	11 11	50 50	18 18	271	271bp CDS (V1 NM_007294.3)
Primer 3 F: 5'ATA TGA CGT GTC TGC TCC AC'3 R: 5'GGG AAT CCA AAT TAC ACA GC'3	20 20	55 55	20 20	401 (NG_005905: 81189bp)	Only 86bp CDS (V1 NM_007294.3)

Figure 1. PCR amplification of *BRCA1* gene. Left-side, PCR amplicons of primer 2, size 271 bp; right-side, PCR amplicons of primer 3, size 401 bp; L, ladder of 100bp each.

tion was at 72°C for 10 minutes⁵¹. After PCR amplification, the PCR products (442, 271 and 401bp) were checked by 2% gel electrophoresis at 100 V for 30–45 min (Figure 1).

The product size of the first and last primers were checked and assessed using Serial Cloner version 2.6.1 (http://serialbasics.free.fr/Serial_Cloner.html) on the known nucleotide database accession gene for *BRCA1* (NG_005905) with the whole sequence size of 81189bp, both forward and reverse of each one have been found to determine regions that cover coding and non-coding sequences.

Reference sequence NCBI nucleotide database

The NCBI RefSeqGene NG_005905, which represents the whole *BRCA1* gene, and the transcript variant 1 NM_007294.3 mRNA, which comprises the coding DNA reference sequence of the gene (cDNA) with 7224 bp that contains the (5'-UTR and 3'-UTR) Untranslated region sequences, and among the sequence bp, the A nucleotide of the ATG initiation codon has been identified to start at 233 and stop at 5824 to determine the coding sequence (CDS) of 5592 bp, which is commonly used in the mutation nomenclature that is supported by the Human Genome Variation Society (HGVS). The first sequence NG_005905 was used to check that all three primers amplicons within the

BRCA1 gene region, while the second sequence NM_007294.3 was used for assessing all the three primers amplicons within the *BRCA1* coding sequence. The gene sequence of *BRCA1* has marked all the primers set amplicons to be within the gene region sequence. The transcript variant 1 (NM_007294) mRNA has marked (only 367 and 86bp) nucleotide sequences within primers sets 1 and 3 amplicons, respectively, to be within coding sequences, and the whole set of primer 2 amplicon (271bp) was within the coding sequence of the *BRCA1* gene (Table 2).

Sequencing of *BRCA1* gene

The PCR products of the 10 best bands yielded from the patient samples for each primer set, a total of 30 resulted amplicons, were sent for Sanger dideoxy sequencing. Partial standard sequencing for the three regions within the gene, including both forward and reverse nucleotide sequencing, was performed by MacroGen Company (Seoul, South Korea), using the same pairs of primers.

Computational analysis

Sequence analysis. The sequence results for the 30 sequence chromatogram files were viewed by FinchTV program version 1.4.0⁵³, which was used to check both nucleotide sequences of the patients forward and reverse sequences to be free of errors. Any errors were excluded during processing. The assessment findings had arrived at higher quality sequencing results with 16/30 sequences to undergo post-sequencing screenings and investigations (Table 3). The Basic Local Alignment Search Tool (BLAST; <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to assess nucleotide and protein sequence similarities⁵⁴. In ExPASy translate tool - SIB Bioinformatics Resource Portal, the gene sequences were translated into amino acid sequences⁵⁵. For primers 2 and 3, the *BRCA1* nucleotide sequences from the patients, with their translated proteins, underwent multiple sequence alignment using BioEdit software version 7.0.9.0⁵⁶. Multiple sequence alignment included the reference sequence with the highest similarity, as obtained by BLAST (RefSeq transcript mRNA - NM_007294 transcript variant 1), two additional nucleotide sequences (NM_007297.3, transcript variant 3 mRNA and JN686490.1; Figure 2A), and the gene sequence NG_005905, which is mainly the sequence between positions 68120-68810 (Figure 2B).

Table 3. The refined patients with mean age 45.4 ± 9.6 years with their sequencing results and validity findings during processing.

No.	Patients	Age (Years)	Primer 1	Primer 2	Primer 3	Valid sequence(s)/ Amplicon(s)
1	2	45	*	-	*	1/2
2	6	40	*	-	-	-/1
3	7	51	*	-	*	1/2
4	10	60	-	*	-	1/1
5	11	48	*	*	*	2/3
6	12	60	*	-	*	1/2
7	21	54	*	*	*	1/3
8	22	40	*	*	*	2/3
9	23	27	-	*	*	2/2
10	24	39	*	-	*	1/2
11	26	37	-	*	*	1/2
12	27	40	-	*	-	-/1
13	29	37	*	*	-	1/2
14	44	57	*	*	-	1/2
15	45	46	-	*	*	1/2
Total valid sequences/Total Amplicons						16/30

(*) showed those sequences that were passed the test validation assessment.

(*) showed those sequences that were excluded after running the test validation assessment.

(-) showed those patients whom were not being selected for this primer.

Figure 2. Highlighting the position of the two variants after assessment. (A) Heterozygous substitution from Glutamine (Q) to Arginine (R) at position 356 in patient 23, due to missense substitution alteration from Adenine (A) to Guanine (G) n.1299A>G, transcript sequence. (B) Heterozygous substitution from Valine (V) to Aspartic acid (D) at position 1736 in patients 2, 22 and 26 due to missense substitution alteration from thymine (T) to adenine (A) n.5439 T>A transcript sequence. Using the Reference transcript variant 1 NM_007294.3 mRNA, which represents the complete BRCA1 transcript (cDNA) sequence, was used to align all patient sequences under screening, and the corresponding Reference amino acid sequence NP_009225, was used to align all patient translated amino acid sequences.

SNP information. SNP information [SNP ID, MIM: 113705, RefSeq Gene accession No.: NG_005905 on chromosome 17, mRNA accession NO.: NM_007294.3 transcript variant 1 with 7224 bp and Protein accession numbers : (NP_009225) protein isoform 1 with 1863 a.a. and P38398 UniProt entries] concerning the human *BRCA1* gene, which was used in our computational analysis, was retrieved from the NCBI database of SNPs: dbSNP (<https://www.ncbi.nlm.nih.gov/snp>).

SNP prediction. SNPs were analyzed using five prediction online tools: SIFT (<http://sift.bii.a-star.edu.sg/>)⁵⁷, Polyphen-2 (<http://genetics.bwh.harvard.edu/pph2/>)⁵⁸, I-MutantDDG-Seq Suite (<http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi>) and PhD-SNP (<http://snps.biofold.org/phd-snp/phd-snp.html>)⁵⁹. The tertiary model of protein and mutation analysis was done online using Hope software (<http://www.cmbi.ru.nl/hope/input>)⁶⁰. MutationTaster2 (<http://www.mutationtaster.org/>) was used to assess the protein features of the resulting variants, with comprehensive (input/output) criteria, which predicts potential disease-causing mutations⁶¹.

Results

Patient sequences

Two missense variants were detected within the study, one patient with Q356R and three patients with V1736D. Both variants were heterozygous (Figure 2) and were detected within premenopausal patients, with a mean age of 37 years. Three patients were multiparous; the one case of (Q356R) and two cases of (V1736D) were multiparous (mean parity, 2.8). There was no family history of BC in patients with the two variants (Table 4).

Bioinformatics assessment

The sixteen patients' sequences, and the additional nucleotide sequences with the gene sequence, were aligned against the reference standard sequence in the RefSeqGene and nucleotide databases (accession, NM_007294.3), which is *BRCA1* gene transcript variant 1, and was introduced to replace the previously existing sequence (gi: 63252871) in May 2009⁶². Two substitutions-bearing monoallelic alterations were found, the first one at position 1299 (A/G) located in exon 11 (Figure 2), and

the second one at position 5439 (T/A) in exon 20 (Figure 2). In conformity with the HGVS nomenclature, the sequence variations 1299 (A/G) and 5439 (T/A) met by (n.1299A>G, c.1067A>G) and (n.5439T>A, c.5207T>A) respectively. The (n) describes the noncoding which highlights the position within the complete transcript that possesses the UTRs where the numbering includes the 5'-UTR, [NM_007294.3:n.1299A>G] and [NM_007294.3:n.5439T>A]. The (c) describes mainly the alteration position within the derived coding sequence that does not possess the UTRs where the numbering starts from the initiation codon ATG, [NM_007294.3:c.1067A>G] and [NM_007294.3:c.5207T>A]. Primer 1 has been excluded from the study because of the errors that have been noticed within all patient sequence data chromatogram results.

After translation to amino acid sequences, the samples were aligned against *BRCA1* protein isoform 1 (accession, NP_009225.1). Q356R was found to meet its corresponding nucleotide change and position n.1299A>G in which Glutamine (Gln) replaced by Arginine (Arg) in patient 23, and V1736D was found to meet its corresponding nucleotide change and position n.5439T>A, in which Valine (Val) was replaced by Aspartic acid (Asp) in patients 2, 22 and 26 (Figure 2). According to HGVS protein nomenclature, both variants will be reported as [NP_009225.1:p.Gln356Arg] and [NP_009225.1:p.Val1736Asp]. These variants (Q356R, V1736D) were then predicted with SIFT, Polyphen-2, I-Mutant-3, PhD-SNP and MutationTaster2 software to obtain their pathological effects, and results are provided in Table 5. Amino acid properties for the wild Val and the mutant Asp residues and the 3D structure of the variant V1736D were obtained using Project Hope software (Figure 3A).

Dataset 1. BRCA1 sequence result in a zipped file

<http://dx.doi.org/10.5256/f1000research.11395.d172445>

These sequencing results as received from MacroGen Company (Seoul, South Korea) comprise all the breast cancer patients in this study using the three sets of primers (1, 2 and 3). Each patient has the sequencing data in different file formats (a sequencing data file that needs to be viewed by a sequencing viewer software, i.e FinchTV; a PDF; and a FASTA format text document).

Table 4. Characteristics and clinical data of the patients with the *BRCA1* variants.

Patient no.	Age (years)	Parturition	Tribe/Residency	Variants	Grading/Staging	Breast cancer type	Family history
2-23	27	Multiparous – 3	Ja'alya/Central-AlGezeerah	Q356R	T ₂ N ₀ M ₀	Unilateral-Right	None
3-2	45	Multiparous – 4	Noba/Western-Kadoggly	V1736D	T _x N _x M _x	Bilateral	None
3-22	40	Multiparous – 4	Kawahla/Central-JabalAwly'a	V1736D	G III	Unilateral-Right	None
3-26	37	Nulliparous–Single	Mahas/Central-Khartoum	V1736D	T _{4b} N ₁ M ₀	Unilateral-Right	None

Table 5. SNP prediction obtained by various sequencing softwares. SNP: single nucleotide polymorphism; **RI**: reliability; **DDG**: $\Delta\Delta G$; **SVM**: support vector; **SVM2 value**: $DDG < 0$: decrease stability, $DDG > 0$ increase stability machine; **DDG value**: DDG (New Protein)- DDG (Wild Type) in Kcal/mole.

SNP	SIFT		Polyphen-2		I-Mutant			PhD-SNP		MutationTaster2	
	Score	Prediction	Score	Prediction	SVM2 prediction	RI	DDG value Kcal/mol	Prediction	RI	Score	Prediction
Q356R	0.01	Affect protein function	0.998	Probably damaging	Decrease protein stability	1	-0.08	Disease-related polymorphism	4	43	Polymorphism
V1736D	0.00	Affect protein function	0.984	Probably damaging	Decrease protein stability	8	-1.82	Disease-related polymorphism	8	152	Disease-causing

Figure 3. 3D structure of mutant *BRCA1* protein due to missense substitution at position 1736 and the structural unit configurations of both wild and mutant amino acid residues of the Q356R variant. (A) This illustration shows the *BRCA1* protein in grey colour on the (left) with the magenta coloured side chain of the mutated residue, and on the (right) the higher magnification of the area of variation displays the side chains of both amino acid residues more clearly, the green highlights the wild type Valine, and the red highlights the mutant type Aspartic acid with their schematic structures, as both contain the same backbone (red) but they differ in their side chains (black). **(B)** The illustration shows the schematic structural units of the neutral wild type Glutamine (left) and the positive mutant Arginine (right). Both amino acids contain the same backbone (Red) but they differ in their side chains (Black).

Dataset 2. Patient demographics (non-identifying) according to primer

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Patient demographics, clinical data, and histological parameters with highlighted detected missense variants (primers 2 and 3) of each 10 selected subset patients.

Discussion

In this study, we found two mutations-bearing monoallelic features. One missense variant rs1799950 in exon 11 *BRCA1* gene in patient 23: position number 1299 (A/G) as (CAG A) to (CGG A), using the complete transcript variant 1 cDNA [NM_007294.3: n.1299A>G], which led to change in the coding amino acid (Glutamine-Gln-Q) to (Arginine-Arg-R) at position 356 using protein isoform 1 [NP_009225.1:p.Gln356Arg] (Figure 2A).

The other was a novel SNP, situated at exon 20, with higher frequency in patients 2, 22 and 26: position 5439 (T/A) as (GTC A) to (GAC A), using the complete transcript variant 1 cDNA [NM_007294.3:n.5439A>G], which led to change in the coding amino acid (Valine-Val-V) to (Aspartic acid-Asp-D) at position 1736 using protein isoform 1 [NP_009225.1:p.Val1736Asp] (Figure 2B). Both SNPs (Q356R) and (V1736D) were found to affect the resulting translated protein. According to Project Hope software, starting with the novel (V1736D) variant, we found that the mutant residue Aspartic acid (D) of the outcome *BRCA1* mutated protein is located near a highly conserved position. The mutant residue is bigger and less hydrophobic than the wild-type residue. The wild-type residue was neutral, the mutant residue is negatively charged. The mutated residue is located on a domain named BRCT which possesses 95 amino acids and lies between amino acid sequence positions (1642-1736) that

is important for binding of other molecules, as it is in contact with residues in another domain. It is possible that the alteration can disturb these contacts. It may also disturb the interaction between these two domains, which would affect the function of the protein; therefore, it might disturb signal transfer from binding domain to the activity domain. This novel variation was detected in patients from different tribes from Sudan, who were all ≤ 45 years old.

In the case of the Q356R variation, the mutant residue of Arg is positively charged compared to the neutral wild type Gln residue. This could lead to the repulsion of ligands or other similar charged residues. The size of the end variant is bigger than the wild-type residue and this might lead to bumps, as reported by Project Hope software (Figure 3B). In addition, the mutated residue is located on a domain named serine-rich which possesses 163 amino acids and lies between amino acid sequence positions (345-507) and responsible for protein activity; hence the activity could be altered by its physical variation conferred by its new charge and size. The Arg 356 variant does, however, generate a run of three positively charged residues (Lys Arg Lys), and as a result it could alter the properties of the protein, which is composed of 16% negatively charged residues overall⁶³. In the current study, we found the same result when it was described as a disease-related mutation of altered protein stability (Table 5).

According to MutationTaster2, the novel variant (V1736D) was predicted to be 'disease-causing', and the software assessed and calculated the probability of such variation on the resulting protein, and showed that the new features would be disease-causing and displayed it with a higher score. By contrast, the software predicted that in the case of Q356R, the resulting Arg was a polymorphism of little harm, and reported it with a lower score compared to the protein feature of the other variant, and reports from the other softwares used for assessment (Table 5). Q356R has not yet been classified in terms of clinical significance in NCBI dbSNP.

Two novel variants were identified within the *BRCA1* gene among premenopausal women patients in two local studies^{9,35}. One variant was c.3999delT, stop codon 1335, a truncated stop codon and the other c.5090G>A, p.Cys1697Tyr, was predicted computationally to alter protein function; furthermore, analysis within control population revealed variant of unknown clinical significance VUS which necessitated the need for further verification. On the other hand, and in the interest of variant's prediction, novelty, solitary existence and being situated at BRCT domain, the study concluded that this variant most likely responsible for BC. In addition to these variants, a deleterious mutation, c.4986+6T>C, located in intron-exon boundary was found in the youngest patient of 25 years in one of the studies³⁵. These findings, compared to our present study finding (V1736D), a variant that needs more exploration, showed that early onset BC is associated with a deleterious nature of the identified variants. In

addition, our study included pre and postmenopausal patients, all detected variants were mainly confined to premenopausal cases. Therefore, genetic studies are highly recommended to highlight the genetically susceptible nature of patients diagnosed with early onset disease, who may harbor the deleterious variants that could have a significant role in developing BC. Two patients within the present study were screened previously in a local study targeting pathological SNPs within *BRCA2* gene selected regions, which identified a stop codon (L1053X)⁶⁴. One of these patients was the youngest patient identified with Q356R, and the other presented with the bilateral disease and identified with V1736D. Both patients were reported to have stop codons at position L1053X with nucleotide and protein sequences identifiers of **KT901810** and **ALQ44030**, and **KT901807** and **ALQ44027**, respectively⁶⁴.

The SNP found in the present study, n.1299A>G, has been highlighted within matched reports as c.1067A>G⁶⁵ and also with similar altered nucleotide position 1299⁴⁴, and the same altered outcome protein position, Q356R^{44,65}. The same protein position (Q356R)⁴² with the same altered nucleotide (A/G) has also been described previously, but with a different nucleotide position 1186 (A/G) which is being more consistent with the nucleotide sequence transcript accession **U14680.1**^{16,66,67}. Some studies found that this SNP is associated with patients under 40 years old^{67,68}, which agreed with our study - the youngest premenopausal patient (27 years old) was detected with this variant. Although this variant's effect remains uncertain, a previous finding to our study has described the same alteration, Q356R in Moroccan BC patients, which was the first study that described this variation in a North African population⁶⁷. They found that most previous studies that described this variation were within western European populations⁶⁹ and there were no studies found in a North African population to confirm this variant association, thus a larger size was recommended to further investigate this change. Our study is closer to the Moroccan study regarding the early onset characteristic this variant has, revealed by the similarity in the background history of the patient with a Q356R polymorphism in our study and those detected in the Moroccan study, who also did not have a family history of BC⁶⁷. This variant has been reported to be independently minor or leads to a very slightly increased BC risk, but a risk that is cumulatively significant⁷⁰. In another study, this variant was found in patients with a family history of ovarian cancer, suggesting that this variant may increase ovarian cancer risk^{66,71}. Both detected variants in the present study were identified to have a pathological effect, with the exception of the results from MutationTaster2 in the case of Q356R. Therefore, theoretically, the Q356R and the novel SNP variants have a prediction to pathologically affect protein function which is in need for further verifications in order to confirm their roles in enhancing the tumorigenesis of the disease practically.

In order to only have clear DNA sequence results for comparison with NCBI references, primer 1 results were excluded due to

sequencing errors (see [Supplementary File 1](#)). In addition, three patient sequences of primer 2 set and one patient of primer 3 set have been excluded for the same sequence errors [Table 3](#).

The limitation of this study was the small sample size and the functional assessment facilities available to assess the protein of monoallelic alteration for their pathological contribution to the disease. Financial constraints also limited the study. Therefore, we recommend further studies in a larger number of Sudanese patients to further explore these findings.

Conclusions

In the present study, Sudanese BC patients were investigated for *BRCA1* mutations. Two different missense variations were found in young patients of ≤ 45 years old, with no family history of BC. To conclude, the study has highlighted a need for further research of these identified variants amongst a larger population (including patients and controls), so as to investigate the variants' distribution through the population and their potential diagnostic value. This will aid the understanding of a variant's frequency and clinical significance. In addition, both variants identified, require *in vitro* functional and protein level assessment.

Data availability

The *BRCA1* sequence data of the novel variant (V1736D) from this study has been submitted to NCBI GenBank under the accession numbers and protein identifiers found in [Table S1](#).

Dataset 1: BRCA1 sequence results in a zipped file.

These sequencing results as received from Macrogen Company (Seoul, South Korea) comprise all the breast cancer patients in this study using the three sets of primers (1, 2 and 3). Each patient has the sequencing data in different file formats (a sequencing data file that needs to be viewed by a sequencing viewer software, i.e FinchTV; a PDF; and a FASTA format text document). doi, [10.5256/f1000research.11395.d172445](#)⁷²

Dataset 2: Patient demographics (non-identifying) according to primer. Patient demographics, clinical data, and histological parameters with highlighted detected missense variants (primers 2 and 3) of each 10 selected subset patients. doi, [10.5256/f1000research.11395.d172446](#)⁷³

Author contributions

MEMMA, AAME, MAS conceived the study. ME-FME, HNA, MMAH, MAS designed the experiment. MEMMA, AAME, sample collection and lab preparation. ME-FME, MMAH designed the wet lab practice. MEMMA, AAME, ME-FME practiced the wet lab methodology. MAS, HNA, AAME, MMG, AAF, MMO, SAO, HAS, MSA, TS, RAO, RAE bioinformatics assessment. AAME, MMG, AAF, MMO, SAO, MSA, TS prepared the manuscript draft. AAME, MMG, MSA, MMAH, MAS revised and edited the manuscript draft. MMAH revised the final edited manuscript.

Competing interests

No competing interests were disclosed.

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Supplementary material

Supplementary File 1: Evidence for sequencing errors of primer 1. The file contains FinchTV snapshots, and the uncropped western blot for primer 1.

[Click here to access the data.](#)

Table S1: NCBI GenBank accession numbers for the novel *BRCA1* sequence data from this study.

[Click here to access the data.](#)

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[Data Source](#)
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[Data Source](#)

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Khalid Dafaallah Awadelkarim

Department of Molecular Biology, National Cancer Institute (NCI-UG), University of Gezira, Wad Madani, Sudan

The reasons of my decision to assign not approved status are as follows:

1. I found it extremely difficult for an illiterate female affected with breast cancer to give an ethical consent for BRCA1/2 genetic testing in the chemotherapy room, combined by the limited time of interaction between researchers and patients, as stated by authors. The author's statements are somewhat troubling. I also reject the author response statement "The patients' care-givers and relatives were not found within the short-stay rooms that were designated only for patients' admissions in order to be prepared and to receive their chemical infusions". Because it suggests that the authors were not willing to move outside chemotherapy rooms/area and get some help from patients' care-givers and relatives to obtain ethical consent from any illiterate patient. Also I reject the all idea about the literacy level, which is in Sudanese women in range of 40%. So by simple math at least 18 out of the 45 patients could have written consent.

Such practices as stated by the authors should not occur and the available local regulations preclude it.
2. Still the manuscript has different numbers to indicate the same change, which I do not approve. The rule of thumb is that any change is always exist in the same chromosomal position and when indicated using coding DNA reference sequences as c.1067A>G or c.5207T>A it should be consistent across the manuscript and all other forms of numbering should be omitted e.g. n.5439T>A, n.1299A>G, 1186 (A/G).....etc. Inclusion of protein nomenclature e.g. p.Val1736Asp or p.Gln356Arg is fine
3. The authors fail to proper document the exact number of patients who produced the BRCA1 data with their characteristics. I found this study is based on the data on Table 3 (15 patients) but in sampling and PCR there is 45 patients. 15 patients were OK giving the research funding constrains in Sudan. 30 cases were excluded from sequencing and did not have BRCA1 status results. This should be clearly stated e.g. 80 year women was not

screened ...etc

4. The p.Gln356Arg has to be classified as polymorphism and I have some concerns regarding the pathogenetic implication of the p.Val1736Asp, because it is a missense mutation detected with high frequency (33.3%; 3/9). The probability of BRCA1 pathogenetic mutation is about 1/1000 and pathogenic variant can be either a nonsense variant, a small insertion or deletion variant that creates a frameshift, a larger gene rearrangement, a variant that creates a severe splicing aberration, or a known pathogenic missense substitution. However, more than 5% of patients are found to carry an unclassified variant (UV)—usually either a missense substitution or a variant that falls in the splice junction consensus regions but outside of the canonical GT-AG dinucleotides. Because of ongoing efforts to classify UVs, most former-UVs with frequencies of above 0.1% have now been classified; consequently, the remaining UVs are individually rare (Vallee HUMAN MUTATION, Vol. 33, No. 1, 22–28, 2012).
5. I reject the author statement that BC is a worldwide issue as a reason to not include the data from Khartoum Cancer Registry and published hospital records from Sudan. The title of the manuscript includes the phrase “in Sudanese patients with breast cancer”. However I am really concerned about the author response statement because they already cited the Khartoum Cancer Registry paper (reference number 12). I asked incidence rate from the reference 12 to replace data from the United States.
6. I do not agree that previous local research finding could justify the adoption of specific molecular screening strategies. In the absence of founder effect the recommended strategy is to screen the entire BRCA1/2 coding sequences, including intron-exon boundaries (complete coverage). When adopting such comprehensive strategy, a note that there are still many mutations will be missed like genomic rearrangements. Instead cost alone could be cited as reason for not performing complete BRCA1/2 screening alongside a note of the above.
7. Still the paper has the PCR figure with a smear. If no good image is available, this figure could be removed as DNA sequence chromatogram were provided. I also agree with Dr. Balbaa that PCR figure with a smear should not be allowed here.
8. All the three primers were selected for their quality performance. Saying the selection was base of primer quality performance seems misleading, given 46.7% exclusion rate due sequencing errors.
9. In the discussion the authors stated that “Two patients within the present study were screened previously in a local study targeting pathological SNPs within BRCA2 gene selected regions, which identified a stop codon (L1053X). One of these patients was the youngest patient identified with Q356R, and the other presented with the bilateral disease and identified with V1736D. This co-occurrence should assist in the classification of BRCA mutations. Because co-occurrence *in trans* with a known BRCA1/2 deleterious mutation is widely considered one of the criteria to designate unclassified variant as neutral.

in trans mean on different copy of the gene which imply both copies were inactive and the well studied examples are embryonic lethality in BRCA1 and Fanconi anemia in BRCA2. To

aid classification Easton et al.¹ assumed that, for *BRCA1*, compound heterozygotes for two deleterious mutations are **vanishingly rare** (occur in the testing population with a frequency of 1 in 10,000), given both biological and genetic evidence that homozygotes or compound heterozygotes for deleterious *BRCA1* mutations are embryonically lethal, whereas, for *BRCA2*, Easton et al. assumed that the probability is 1 in 1,000, the increased frequency reflecting the fact that viable compound heterozygotes have been reported as Fanconi anemia, type D1.

The frequency of two deleterious mutations (with one in *BRCA1* and one in *BRCA2*) is also rare. Since Ramus et al.² first report a patient with two deleterious mutations (with one in *BRCA1* and one in *BRCA2*), subsequent case reports mostly have been in Ashkenazi populations (Friedman et al.³; Liede et al.⁴; Frank et al.⁵). Frank et al.⁵ found 10 patients with both mutation in *BRCA1* and *BRCA2* of 10,000 patients, which was significantly lower than expected 30 patients from an equilibrium population of 2,539 Ashkenazi women. Friedman et al.³ also found only three patients of approximately 1,500 breast or ovarian cancer patients, and they postulated that there may be some selection against mutations in each *BRCA1* and *BRCA2*.

However it is the responsibility of the authors to update/recheck the above-mentioned and try finding some working ways to articulate their finding of two patients having two mutations (one in *BRCA1* and one in *BRCA2*) out of 15 patients (13.3%). If both were assumed deleterious then the authors have to comment and/or settle this versus the extremely infrequent such phenotype i.e. 0.1% (10/10,000) and 0.2% (3/1,500) in Ashkenazi populations.

Otherwise owing the above-mentioned rare probabilities of double mutant and assuming *BRCA2* L1053X alone as deleterious mutation both Q356R and V1736D will be classified as neutral based on the reported co-occurrence with the nonsense *BRCA2* L1053X. If *BRCA2* L1053X was not assumed as deleterious why included or even bother to mention?

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Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 29 Jan 2018

Alsmawal Elimam, Postgraduate College, Al-Neelain University, Khartoum, Sudan

Dear Dr Khalid, you very quickly responded with your decision.

1. We were there at the time of sampling the first and the second authors; we were more concerned about the status of the patients than to obtain the written consent, as the way you described it "I found it extremely difficult for an illiterate female affected with breast cancer to give an ethical consent for BRCA1/2 genetic testing in the chemotherapy room" while the nurses were preparing the patients we had arrived earlier at the early morning to have them at our side, they helped us a lot to establish our work, then we asked those patients affected with BC individually with a note book to record every important data said by the patients, we asked them to participate in our study, in the light of their agreement, blood samples have been drawn through their butterflies that had been fixed on their veins prior infusion. They were happy to participate, we were sad; we have been completely filled with sorrows during this part of the work. We were there in Almal tower-the Fifth floor by our existence and acting the reality of those events and we made it possible through the light of this work.
2. The ways that described the two findings were supported by HGVS and the whole aspect has been governed.
3. The all parts of the present study were filled with motives, encouraged spirits, and humanitarian interactions which led all of that to participate within the Article (texts, tables and figures) in which the sampling and the PCR both represented the true and real practices and left true humanitarian and scientific impact to this Article. Science and humanity are always tightly bound together.
4. We made together these predictions terminology, because we believed in our work with greater respect to the literature (was controversial) and that the work was describing more the finding suggestively rather than confirmatively. The novelty had a frequency based on a very little sample size (more size was requested). This aspect has been governed too in our previous response Notes (4, 5 and 7).
5. The US was highlighted very recent statistics regarding BC (2014 and 2016) while we lacked the recent statistics and when considering that such different ethnicities within US, as there always African-American numbers existed with other found such relocating ethnicities the way you might describe a little world/glob ethnicities within these statistics which acted to represent the world and African women with breast cancer and as a very necessary recent substitution. Please also refer to Note (8).
6. Elnour, et al.2012 [10] possessed our adoptive justification; the study has highlighted and made it easily to follow the most regions frequently subjected to receive variations within Elnour's study and advising to put these regions with great care in the future which offered a great chance which means a great beginning success to establish a respectful work scientifically. Based on local literature we originated this

Article and we are so proud of that.

7. Please refer to note (3) in addition; this aspect has been governed in our previous response Note (11).
8. This has been governed through our previous note (18) in respect to your notice. In addition, they were represented our three regions basis of selections, so they were selected from literature on behalf of the Local findings, the cost and oligoanalyzer guidance.
9. More deepening, deficit – *BRCA1* disturbs chromosomal integrity and genomic stability by which increases the mutation rate of other/all genes [25]. Please also refer to Note (21).

Elimam

Competing Interests: No competing interests were disclosed.

Version 3

Reviewer Report 18 December 2017

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Khalid Dafaallah Awadelkarim

Department of Molecular Biology, National Cancer Institute (NCI-UG), University of Gezira, Wad Madani, Sudan

Dear authors,

This manuscript will benefit from more additional revision and amendments as follows: -

1. The usage of HGVS nomenclature was not done in a correct way. Please refer to the following links: <http://varnomen.hgvs.org/recommendations/DNA/variant/substitution/> ; <http://varnomen.hgvs.org/bg-material/numbering/> ; <http://varnomen.hgvs.org/recommendations/protein/variant/substitution/> ; Simply, the authors did not follow the HGVS guidelines instead they added more numbers to what they already have (lot of numbers that bring lot of confusion). Please note that the current version of HGVS nomenclature is v15.11, which means that previous published work follow different nomenclature rules or simply do not follow previous HGVS nomenclature. Generally, the use of different nomenclature parameters, will not generate different or altered nucleotide position as the authors stated in the discussion section (The SNP found in the present study, n.1299A>G, ..., but with a different nucleotide position 1186 (A/G) which is being more consistent with the nucleotide sequence transcript accession U14680.1). At

the end it is the same nucleotide at the same position with the same predicted effect. And when following HGVS nomenclature the use of only one numbering method with only one Reference Sequence is mandatory. Subsequently, the discussion would be for the findings only not for numbering systems. If after making the necessary normalization, a mutation was found to be in a different position, then it will constitute a different mutation. The use of only one Reference Sequence is mandatory, to avoid confusion and to make it easy for others to track the documented mutations (in this case either NG_005905 or NM_007294). In this regards, please note that the coding sequence in the GenBank: U14680.1 start at 120 and the 119 constitute the 5'UTR (un-translated region) and the coding sequence in the NCBI Reference Sequence: NM_007294.3 start at 234 and the 233 constitute 5'UTR.

2. When applying the above-mentioned criteria to the NCBI Reference Sequence: NM_007294.3 as the authors stated, I found that the reported novel mutation (V1736D; n.5439 T>A), would be *BRCA1* c.5206T>A of the reference sequence NM_007294.3 not c.5207T>A. Note that at position 5207 there is a c "Cytosine". (5401 taaagaaaga aaaatgctga atgagcatga ttttgaagtc agaggagatg tggtaatgg)
3. The 5206T>A was previously described in breast cancer patients from China¹. Hence it is important to verify this before judging this variant as previously novel/unpublished/unreported.
4. The status of the Q356R is still the same without further evidence to support pathogenetic effect against the vast majority of evidences to be classified as polymorphism as in the cited references in the first peer-review report. The authors relay only on bioinformatic tools which was widely recommended that "these programs should not be used to decide, in the absence of other tests or arguments, whether the sequence variation found in a patient is or is not responsible for the disease"².
5. No screening in control population for V1736D variant was conducted. This variant was detected with a frequency of 3/9 (33.3%, one patients was excluded for sequence error) and regarded as a pathogenetic mutation based only on bioinformatics studies. Which is not acceptable giving the claim of pathogenetic role, novelty "without previous reports to be cited" and its high frequency (3/3, 33.3% occurrence).
6. In many part of the manuscript there is many comments (The mutated residue is located in a domain), which domain and what is the predicted change on function? Was not resolved and still need amendments!
7. The introductory part about SNPs page 3, third paragraph, second sentence. Was partially re-written but still very hard to follow and without clear focus i.e. SNPs approach or mutation screening these two concepts intermingle randomly across the manuscript. The germline mutations have to be mentioned first, SNPs would be next.... The rs1799950 part is not really part of the introduction and the entire section about the rs1799950 has to be moved to the discussion section.
8. In the introduction part the authors stated that "In the US, it was estimated that there were approximately 232,670 new cases of BC and 40,000 BC deaths in 2014" is not relevant and should be replaced with the data (incidence rate, prevalence.....etc) from Khartoum Cancer

Registry³ and data (percentage and total cases reported) from published hospital records from Sudan (reviewed by Awadelkarim, K.D. *et al.*⁴) and data from other African countries (similarities and dissimilarities) with specific emphasis on Sub-Saharan Africa, including Nigeria, Chad, Sudan, Cameroon, Central African Republic, Niger, Namibia, Congo, Kenya, and Somalia⁵. This concord with the peer-review report of Dr. Chaabouni-Bouhamed, which I think went without response.

9. In the introduction the authors stated that “Two genetic studies have been conducted in Sudan concerning BRCA1” and mentioned three studies. Please correct. Also a detailed mention/description of the mutations reported is mandatory i.e. the type, name, frequency, designation (neutral, pathogenetics, unknown significance).
10. In the introduction the authors stated that “These regions and their selections were based on the quality of available primers (e.g. best GC content, adequate length, according to previous literature), previous local research findings revealing frequent mutations within exon 11, and the cost.). I think this phrase should be omitted. Because it is misleading; first the primers were not of good quality as stated (Primer 1 was excluded, three patient sequences of primer 2 set and one patient of primer 3 set were also excluded because of sequence errors, Figure 1 with evident PCR smear), second only founder mutation could justify the adoption of specific molecular screening strategies⁶, and in the absence of such founder effect the recommended strategy is to screen the entire BRCA1/2 coding sequences, including intron-exon boundaries (complete coverage). When adopting such comprehensive strategy, a note that there is still many mutations will be missed like genomic rearrangements (that could contribute to the homozygous BRCA1/2 variants observed in the series), mutations in regulatory sequences and epigenetic alterations is warranted.
11. Still the paper has the PCR figure with a smear. If no good image is available, this figure could be removed as DNA sequence chromatogram were provided. Not resolved! I also agree with Dr. Balbaa that PCR figure with a smear should not be allowed here.
12. The actual laboratory work need (Methods section) to be re-written with more clear and straightforward approach ..., 45 blood samples collected and DNA extracted. Three sets of primers were used. So I expect about 3X45 sequences (135 PCR and sequencing products), but learned later that 10 samples were selected for each primer (30 PCR products), and later learned that Primer 1 was excluded from the study (Page 7 second column, first paragraph, line 1) for errors that have been noticed with all patients sequence data. My understanding now changes that this study is based on 20 PCR products that was sequenced to generate the results. But learned later at the end of the discussion that three patient sequences of primer 2 set and one patient of primer 3 set were also excluded because of sequence errors, and my understanding changes again that the total is 16 PCR products (20-4=16), which were sequenced to generate the results. The exclusion rate is 46.7% (14/30) for primers; what is the exclusion rate for patients (45 patients collected and DNA extracted)? Saying the selection was base of primer quality seems odd, given 46.7% exclusion rate due sequencing errors. In short, I found that the conducted work was on 16 PCR products, which could be OK, but has to written this way from start and in all related sections (Abstract, Material and Methods ...etc). Another question from mutation screening point of view; does each patient have two amplicons? Or the selection was random leading to more

than 10 patients in total, with some having two amplicons and others only one amplicon? This has to be clearly stated.

13. Why the excluded primer (Primer 1) still have all the computational analysis documented.
14. In Figure 3 nonsense should be changed to missense.
15. The notion in the discussion that the c.5090G>A, p.Cys1697Tyr was predicted computationally is not entirely correct because this variant was analyzed in a control population of 180 individuals (111 males and 69 females) and was classified as variant of unknown clinical significance (VUS) (Table 1, Biunno I, *et al.*⁷) and these authors conclude that the p.Cys1697Tyr is most likely responsible for BC in a very lightweight form "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".
16. The discussion of the previous detected variants the authors stated the "Two novel variants were identified to be deleterious" and mention three, please correct. Citing Awadelkarim KD, *et al.*⁸ and Biunno I, *et al.*⁷. What about the findings from the previous cited reference Elnour AM, *et al.*⁹. Indeed Elnour AM, *et al.* was not able to name and/or did not report any specific BRCA1.2 mutation and should be discussed accordingly.
17. The mean age has to have standard deviation (SD). What are the cutoff used for early onset and late onset breast cancer? Why a women with an age of 80 years got BRCA1 mutation screening, because rates of *BRCA1* and *BRCA* mutation testing are increasing in young women (younger than 40 years)¹⁰ and the National Comprehensive Cancer Network (NCCN) guidelines recommend that women diagnosed as having breast cancer at 50 years or younger undergo genetic testing (National Comprehensive Cancer Network (NCCN) clinical practices guidelines in oncology: genetic/familial high-risk assessment: breast and ovarian. Version 2.2015.
http://www.nccn.org/professionals/physician_gls/pdf/genetics_screening.pdf)
18. All the three primers were selected for their quality performance, what does this mean? Which are the parameters? Primer 1 was excluded? Saying the selection was base of primer quality performance seems odd, given 46.7% exclusion rate due sequencing errors.
19. The product size of the first and last primers were checked and assessed using Serial Cloner version 2.6.1 (http://serialbasics.free.fr/Serial_Cloner.html) on the known nucleotide database accession gene for *BRCA1* (NG_005905) with the whole sequence size of 81189bp, both forward and reverse of each one have been found to determine regions that cover coding and non-coding sequences; could be deleted as it seems out of context, but if the authors feel it has to stay in the paper please move it to the bioinformatics section.
20. The PCR products of the 10 best band yielded from ....etc. which are the parameters? Exclusion criteria?
21. In the last part of discussion the authors stated that "Two patients within the present study were screened previously in a local study targeting pathological SNPs within *BRCA2* gene

selected regions, which identified a stop codon (L1053X). One of these patients was the youngest patient identified with Q356R, and the other presented with the bilateral disease and identified with V1736D. This co-occurrence should assist in the classification of BRCA mutations. Because co-occurrence in trans with a known BRCA1 deleterious mutation is widely considered one of the criteria to designate unclassified variant as neutral¹¹⁻¹³ and should be discussed accordingly.

22. I have some concerns regarding the phrase “the literacy levels of the patients”. Because most, if not all, of the patients come with care-givers who could help in obtaining ethical consent, and the literacy level in Sudanese women is in range of 40%. Please re-write/re-consider.

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Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 19 Jan 2018

Alsmawal Elimam, Postgraduate College, Al-Neelain University, Khartoum, Sudan

Dear Referee 3: Dr.Khalid Dafaallah Awadelkarim

Thank you for your valuable continual support and efforts through additional notes and recommendations

Important notes to be considered in response to your last notes and recommendations:

1. Although Human Genome Variation Society nomenclature has been followed in order to provide more consistency with the literature, the later filled with different mutation nomenclatures, whether the HGVS has been followed or not, most of that could be attributed to the nucleotide reference sequence variations describing the same alteration with nucleotide position variation while the protein still intact to act more specifically to describe the change. The differences mostly have been observed came as a result of the nucleotide alteration in positions as mentioned above could be attributed to, i.e. *BRCA1* nucleotide accessions include six known variants transcripts (NM_007294, NM_007300.3, NM_007297.3, NM_007298.3, NM_007299.3 and NR_027676.1) in addition to the U14680, they posses different size bp and this could explain the variations in the nucleotide position of the same variant among the literature. HGVS assisted to minimize such variations by designing its way to unify variant description putting into consideration the main clarification of the variant complete description will be against the related, used reference sequence that must be documented and highlighted clearly. When the RefSeq first being identified then the finding will be more comparable and discussable. Besides, the Reference sequence complete transcript variant 1 has been used to describe the findings of the present study in both, the nucleotide positions, and the new mutant types (i.e. n.1299A>G and n.5439T>A) and it has protein isoform 1 [NP_009225] which describes these sequence variations very specifically upon the corresponded amino acid change and position of the new mutant type through literature in more convenient way. Diversity has arisen and originated from within HGVS nomenclature, specifically on nucleotide changes, to be followed or not, the literature covered with them, in that the one RefSeq could have two nomenclatures, here we settled on the one that identified the study findings (the complete transcript) but shall always be of value to touch it, highlighting the variations among the CDS sequence (i.e c.1067A>G and c.5207T>A), where the supported information being both available to stay away from

confusion. Variants distributed within literature (i.e 1186, 1299 and 1067) to highlight most of them in one place providing an example of each change and the reasons behind such variations why exist will be very informative. The Reference Sequence NG_005905 which represents the whole *BRCA1* gene has been used to highlight the non-coding regions within primer 3 nucleotide sequences. And have been used to determine the amplicon of primer 1 set. But all mutations at the present study have been identified and nomenclated according to variant 1 RefSeq. The start codon of the complete sequence transcript [NM_007294] ATG starts at 233 in which the Adenine is situated at position 233. At the NCBI nucleotide database – the reference sequence variant 1 page within the site. The CDS and the sequence to cover were both being highlighted under sequence FEATURES. The CDS will lead to a FASTA format that free of both UTRs. This sequence CDS FASTA has been used to identify the two variants of the present study in order to fill the appropriate data required by the HGVS nomenclature in your previous recommendation and to maintain diversity within HGVS nomenclature compared with what the study could have of such touch, which could leave more strengthened imprints. Mutalyzer is an online multi-tasks tool which one of these tasks, the program designated to display sequence variation according to HGVS nomenclature (<https://mutalyzer.nl/>). HGVS protein nomenclature has been expressed.

2. The variation identified within exon 20 was the nucleotide substitution of the normal Thymine with the mutant Adenine in three patients, using the complete reference sequence mRNA transcript variant 1 [NM_007294] that possessed the both UTR regions, then the only coding sequence CDS of the same complete transcript was yielded the same nucleotide type alteration. So the variant n.5439T>A is according to the complete transcript and the same variant will be c.5207T>A according to the CDS of the complete transcript.
3. The mutation you have shed the light on (5206T>A) was found within exon 18 as the article documented. According to the complete transcript variant 1 [NM_007294] exon 18 lies between (5307..5384) cDNA and (5075..5152) CDS, which indicates clearly this position (5206) of the nucleotide Thymine is inconsistent with the RefSeq complete transcript exon 18 sequence. Searching has been performed among other complete transcripts and discovered that this exon is more consistent with the complete transcript U14680 in this position 5206 in which in this sequence accession: exon 18 lies between sequence positions (5194..5273) of the complete transcript U14680 and (5075..5154) of the CDS. Using the same accession: the description for this mutation (5206T>A) will be [U14680 CDS: c.5087T>A] [U14680 cDNA: n.5206T>A]. The corresponded nucleotide position alterations of this variant (5206T>A) according to the Variant 1 will be (NM_007294 CDS: c.5087T>A) (NM_007294 cDNA: n.5319T>A) to be both included within exon 18. Moreover, at codon level the change will be (GTG>GAG) to result in protein variation as (V1696E) with the SNP id rs397509226. (<https://www.ncbi.nlm.nih.gov/clinvar/20921893/>)
4. About the Q356R status, although the mutation has been found within control group more frequently in its heterozygous state which indicates its benign effect on the disease, but on protein functional assessment the heterozygous status has been confirmed to confer altered cellular behavior [45, 46]. Besides, some articles found this variant in its homozygous status (RR) more frequently among familial BC cases comparing to controls [43, 44]. Herein, we highlighted a little review among literature

- variation findings of this variant rs1799950 and presented them in one place to be more informative Article and to highlight the present finding in a bioinformatics way.
5. The study used a prediction terminology (the bioinformatics way of expression) to describe the finding rather than deciding it as true pathogenic or making a confirmation. As guidance to follow in the future assessment (could originate an established hypothesis).
  6. The novel V1736D is located in a domain named the BRCT domain in C-terminal region. The Q356R is located in a domain named serine-rich domain. These information supported by the online Hope after the variation assessment report has been made [60]. The information about either domain (name and more description) has been added where those domains were mentioned within the Discussion.
  7. The SNPs information has been gathered from the NCBI-SNP database. The site included every SNP with its SNP id, in which germline mutations were part of that. ##Based only on bioinformatics with very little sample size and such results against studies with bigger sample size and more assessment facilities will be incomparable to discuss. So to provide a guide with the necessary information about this alteration will find its value much more within the introduction.
  8. BC is a worldwide issue. For the African statistics, please refer to the Reference data [3] within the Article (Ferlay, *et al.* 2013) supported by the most recent GLOBOCAN 2012.
  9. The other genetic study has been highlighted in a separate paragraph for that the study mainly screened *BRCA1* point mutation. The two genetic studies have screened both *BRCA1* and *BRCA2* in which the later has been added according to your notice.
  10. In order to be followed with great respect, step by step, scientifically we originated, actualized and made it possible. The oligoanalyzer is online software assisted in primers adequacy measurements among standard well known measures (GC content, primer length).The cost, local literature and primer quality determined the three regions.
  11. The Macrogen Inc. Company used to purify the PCR products prior dideoxy sequencing.
  12. They all represent essential parts of this work and have been written appropriately according to the subsection headings– sampling, DNA extraction and PCR amplification. Errors were noticed earlier after chromatogram check through FinchTV v 1.4.0 within the computational analysis subsection of the Methods, and has been highlighted in general context (Any errors were excluded during processing) and where found necessary – Result, the Bioinformatics assessment subsection and at the end of the Discussion before the last paragraph. Before errors have been checked and after, which left clear and appropriate explanations at either site. A table (Table 3) has been made to include patient sample sequencing result validation findings with description to their PCR amplicons. The table cited the first paragraph (sequence analysis) to include the first three sentences. Then the refined valid sequences number was highlighted at the beginning of the Bioinformatics assessment subsection of the Results. In addition, Figure 2 highlighted through (the multiple sequence alignments illustration) the entire post-sequencing sequences assessment with patient ids.
  13. Primer set 1 had sequencing results, and has been highlighted before errors detection and after where found appropriate and necessary to document its

existence as one of the essential part forming the basis of this work, and clearly its omitting/exclusion after errors were highlighted. The errors were highlighted within the computational analysis subsection of the Methods, then all the rest of mentioned primers sets were free of primer set 1 and confined mainly to primers sets 2 and 3. The only phrases contained the primer set 1 after the errors were identified to mainly confined to two places where they highlighted its exclusion, in the Bioinformatics assessment subsection of the Result and at the end of the Discussion before the last paragraph.

14. The requested correction has been fulfilled.
15. Which one, VUS or most likely responsible for BC? Both have been added in accordance with the Article (Biunno I, et al.2014) [35]: Information has been added to highlight the control population assessment result finding with the need for further verification as recommended within the text (Biunno I, et al.2014) [35]. Moreover, the confirmation of this variant to be pathogenic and responsible for BC was based on in silico assessment, novelty, solitary occurrence, and for being at BRCT domain – also has been mentioned.
16. Two novels and additional pathogenic variant within this paragraph and the phrase “were identified to be deleterious” has been edited according to your previous recommendation note (15). What about (Elnour, et al. 2012) [10]? Elnour Article findings have been stated clearly throughout the present work where found appropriate and necessary and indeed were of great values to aid in guiding the present study work alongside the other local literature.
17. As recommended, the mean age STD deviation has been expressed. The cutoffs for both statuses were shown within the Table 1, (≤ 45) for the early onset, (≥ 46) for the late. The selection was random (any case diagnosed with BC), and was more convenient. In addition, the random selection shed the light on the frequency of both groups. This was advantageous.
18. The oligoanalyzer is online software was used to assess primer effectiveness among standard well known measures (GC content, basepair length, Melting temp. (Tm)) against enormous published primers sets within one Article (Dufloth RM, et al. 2005) [51]. Variables standard measures that were used in primers selection (primer length between 18 to 24 bp) (GC content 40 to 60%) and the Tm always relays on the GC content and the length of the primers within the range (52 to 58 °C).Primer 1 set recorded readings in which the forward gave a report of 40% GC content, 20 bp primer sequence length and 49.3 °C Tm. The reverse gave a report of 45% GC content, 20 bp primer length and 50 °C Tm. Annealing temperature of both (F, R) have been calculated according to the purchased primer (F, R) Tm.
19. This information was describing more (Table 2) and will be moved as additive note related to the table.
20. The most identifiable bands; those identified with weak bands did not have any priority of selection over the best looking bands for DNA sequencing.
21. In the light of our finding as absence of established deleterious BRCA1 variant in the present study, this term co-occurrence is far from describing this situation.
22. The patients’ care-givers and relatives were not found within the short-stay rooms that were designated only for patients’ admissions in order to be prepared and to receive their chemical infusions.

Competing Interests: No competing interests were disclosed

Version 2

Reviewer Report 23 October 2017

<https://doi.org/10.5256/f1000research.13766.r26925>

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Khalid Dafaallah Awadelkarim

Department of Molecular Biology, National Cancer Institute (NCI-UG), University of Gezira, Wad Madani, Sudan

This manuscript is emphasized in a good way. However, it will benefit from some more revisions.

Major points:

1. I recommend the usage of standard HGVS nomenclature for documenting the detected variants. This will resolve the issue of different nucleotide position for the Q356R variant. Moreover, the novel V1736D need to be re-checked for its novelty after applying the standard HGVS nomenclature.
2. The Q356R is widely regarded as polymorphism (Borg et al. (2010)¹), or at least of very minor risk. What the additional evidence provided by this paper giving it's the evident lack of control population and functional study. I suggest designating this variant as polymorphism or at least as unclassified variant based on the bioinformatics tools. However, Hadjisavvas et al. (2002)² suggested that S1512I in combination with Q356R may be disease-associated, since these two missense mutations are simultaneously present only in this family, and in none of the other samples from the breast cancer or control groups.
3. The V1736D variant need to be investigated more, at least in control populations. I seems to me it is very hard to say that a novel variant that is detected with a frequency of 3/10 (30%) is probably a pathogenetic mutation based only on bioinformatics studies. Thus verification on control populations will provide more information about this variant.

Minor points:

1. In many part of the manuscript there is many comments (The mutated residue is located in a domain), which domain and what is the predicted change on function?

2. The introductory part about SNPs page 3, third paragraph, second sentence “These” I think refer to BRCA1 mutations, whereas the paragraph about SNPs. This is mainly because frameshift mutations are not part of SNPs. Please resolve.
3. The PCR figure appears to have a smear. If no good image is available, this figure could be removed as DNA sequence chromatogram were provided.

References

1. Borg A, Haile RW, Malone KE, Capanu M, et al.: Characterization of BRCA1 and BRCA2 deleterious mutations and variants of unknown clinical significance in unilateral and bilateral breast cancer: the WECARE study. *Hum Mutat.* 2010; **31** (3): E1200-40 [PubMed Abstract](#) | [Publisher Full Text](#)
2. Hadjisavvas A, Adamou A, O'Dowd Phanis C, Todd C, et al.: Q356R and S1512I are BRCA1 variants that may be associated with breast cancer in a Cypriot family. *Oncology Reports.* 2002. [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 09 October 2017

<https://doi.org/10.5256/f1000research.13766.r26244>

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Mahmoud Balbaa

Department of Biochemistry, Faculty of Science, Alexandria University, Alexandria, Egypt

Figure 1 is still in a bad quality and should be changed to show clear band with a bp scale. The band of housekeeping gene should be shown in the figure.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Enzymes and cell signalling, protein expression, gene expression

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Version 1

Reviewer Report 14 September 2017

<https://doi.org/10.5256/f1000research.12300.r25416>

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Mahmoud Balbaa

Department of Biochemistry, Faculty of Science, Alexandria University, Alexandria, Egypt

BRCA1 was highly investigated around the whole world. The authors just applied the research of BRCA1 on Sudanese patients. Generally, the authors have obtained good results, but Figure 1 that shows the PCR amplification of the BRCA1 gene has a bad quality and should be changed to show clear band with a bp scale. The band of housekeeping gene e.g beta actin should be shown in the figure.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.**Reviewer Expertise:** Enzymes and cell signalling, Protein expression, gene expression.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 04 September 2017

<https://doi.org/10.5256/f1000research.12300.r24993>

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Habiba Chaabouni-Bouhamed¹ Laboratory of Human Genetics, Faculté de Médecine, University Tunis El Manar, Tunis, Tunisia²

Centre Urbain Nord, Tunis, Tunisia

The authors report the molecular analysis of 2 exons (11 & 20) of *BRCA1* in a Sudanese population. The paper is well written.

It gives a truncated information about *BRCA1* mutations in a population but the work is correctly done; it has to be completed by analysing the whole gene; using HRM method could help them to reduce cost analysis.

One remark about reference 1 & 2, I didn't find information about African statistics which are available in references 4 & 5.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Genetics, molecular genetics, cytogenetics, genetic counseling

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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