RESEARCH

BRIP1 overexpression is correlated with clinical features and survival outcome of luminal breast cancer subtypes

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Abstract

In Oman, breast cancer is most common, representing approximately more than 25% of all cancers in women. Relatively younger populations of patients (25–40 years) present surprisingly with an aggressive phenotype and advanced tumor stages. In this study, we investigated differential gene expressions in Luminal A, Luminal B, triple-negative and Her2+ breast cancer subtypes and compared data to benign tumor samples. We identified a potential candidate gene *BRIP1*, showing differential expression in the four breast cancer subtypes examined, suggesting that *BRIP1* has the profile of a useful diagnostic marker, suitable for targeted therapeutic intervention. RT-qPCR and Western blotting analysis showed higher *BRIP1* expression in luminal samples as compared to triple-negative subtype patient's samples. We further screened *BRIP1* for eventual mutations/SNPs/deletions by sequencing the entire coding region. Four previously identified polymorphisms were detected, one within the 5′-UTR region (c.141-64G>A) and three in the *BRCA*-binding domain (c.2755T>C, c.2647G>A and c.3411T>C). Kaplan–Meier analysis revealed that patients with overexpression of *BRIP1* displayed a poor survival rate ($P<0.05$). *BRIP1* has a dual function of an oncogene and a tumor suppressor gene in addition to its role as a potential biomarker to predict survival and prognosis. Data obtained in this study suggest that *BRIP1* can plausibly have an oncogenic role in sporadic cancers.

Key Words
- breast cancer
- *BRIP1*
- oncogene
- overexpression
- biomarkers

Endocrine Connections (2018) **7**, 65–77

Introduction

Breast cancer is the most common among women, affecting 25% of female population worldwide and more than a million new cases are diagnosed every year (1). Environmental components are considered serious risk factors, whereas genetic factors remain modest and contribute to only 20% of the cases. In Oman, a Middle Eastern country (2), breast cancer is the most common cancer among women and represents about 25% of all females' cancers (3), affecting frequently a relatively younger Omani population (~67% are below the age of 50 years) as compared to women in other parts of the world (4).

The rapid advancement in molecular biology-related technologies has contributed significantly to our understanding of mechanisms underlying the setup of breast cancer. A panel of relevant genes suspected to

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https://doi.org/10.1530/EC-17-0173

play a key role in the pathogenesis of breast cancer was identified, and their mutations being implicated in the development of breast cancer. These include p53, *BRCA1* and *BRCA2*, *ATM* and *PTEN*; all found to be associated with breast cancer. Screening for germline mutations in these genes, especially *BRCA1* stands out as the major breast cancer gene commonly used in the diagnosis of breast cancer (5). Nonetheless, breast cancer remains a complex disease involving several factors, and therefore, may involve a cocktail of genes alterations contributing to the onset and progression of breast cancer. Although, several genetic alterations have been identified in breast cancer, the frequency of different gene aberrations remains however quite low due to either oncogene amplifications or tumor suppressor gene (TSG) mutations/deletions (6).

Among the frequently mutated oncogenes, *HER2* (20–30%), *c-MYC* (1–94%), *RAS* (<5%), *Cyclin D1* (>50%), *Cyclin E* (13–20%) and *Estrogen receptor* (*ERα* and *ERβ*) (60%) were reported, whereas *BRCA* genes (*BRCA1* and *BRCA2*) (40–80%), *RB* (30%), *TP53* (56–90%) and *PTEN* (25–50%) are the tumor suppressor genes (TSG) reported to be implicated. Mutations in the above genes, especially *BRCA1/2* in the majority of sporadic cases are rare and fail to explain these cases (7, 8), involving somatic mutations or variants of low penetrance sequences in a particular genetic background. Furthermore, several studies showed that lack or low expression levels of BRCA protein play a crucial role in the development of sporadic breast tumors (9, 10). Based upon the relative risk, breast cancer predisposing genes are categorized into high (*BRCA1/2*, *TP53*, *PTEN*, *CDH1*), moderate (*ATM*, *CHEK2*, *BRIP1*, *PALB2*) and low (*MAP3K1*, *FGFR2*, *LSP1*, *CASP8*) penetrance genes (11, 12).

Interestingly, in Oman, breast cancer diagnosis, in general, reveals aggressive subtype and advanced stages (stage III or IV) of breast tumors, likely due to either biological aggressive subtypes or a low index of suspicion and delayed diagnosis (4, 13). Breast cancer affecting younger female population remains intriguing; <50 years compared to 63 years for counterparts in the West (14, 15). This could be explained by a complex interaction between genetic background and environmental factors (16), which is consistent with a previous Lebanese and Saudi study (17), indicating higher proportion of young breast cancer in the Middle East when compared to Western countries. Usually, breast cancers in younger population tend to be more aggressive in nature and are associated with unfavorable prognosis when compared to women having the disease at later stages (15, 18, 19, 20).

Based on the above observations related to sporadic cases, as well as the lack or the low *BRCA1/2* expression, these sporadic cancers could possibly have the *BRCA*ness phenotype (8, 21). Therefore, we hypothesized that a subset of genes are directly or indirectly involved with BRCA proteins during the transition from the normal to the cancerous phenotype. We investigated gene expression differences in human breast cancer and identified genes with a role in the onset of breast cancer either independently or in association with *BRCA1/2*, such as the role played by genetic modifiers (22) that could represent useful diagnostic markers or targets for therapeutic purposes. One potential candidate gene interacting with *BRCA1/2* to regulate DNA repair and cell cycle was identified as *BRIP1* gene, displaying 5-fold expression in the breast tumors as compared to the normal/benign tissue.

Materials and methods

Subject and sample collection

This research was approved by the local Research Ethics Committee at the Sultan Qaboos University (SQU), and written informed consent was obtained from each participant.

Following a predefined protocol, we recruited 50 BC patients, and 30 healthy individuals and/or carriers of benign tumors from January 2012 to April 2014 underwent biopsy and breast cancer surgery. Breast cancer was confirmed by biopsy reports as well as pathologically by molecular tests including estrogen receptor (ER), progesterone receptor (23), Her2 and Ki67 tests in addition to a histological examination using immunohistochemistry. Her2 status was determined using fluorescence *in-situ* hybridization (FISH). Breast tissue samples were collected during biopsy and mastectomy/lumpectomy in cryo-vials containing RNA*later* solution (Ambion, ThermoFisher Scientific) and stored at –80°C until use.

Based on the patient information, the cases were classified into the four molecular subtypes as Luminal A, Luminal B, Her2+ and triple-negative BC cases.

Patients below the age of 10 or above 90 years, who had undergone previous chemotherapy, radiotherapy or mastectomy, and had chronic use of corticosteroids or non-steroidal anti-inflammatory drugs, were excluded from this study.

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Cell lines and tissue culture

Two different breast cancer cell lines (MCF-7 and MDA-MB-231) derived from females were purchased from American Type Culture Collection (ATCC) and were investigated for their *BRIP1* expression. These cells represent different grades and subtypes of breast cancer; MCF-7 represents the luminal subtype, while MDA-MB-231 represents the triple-negative subtype (1, 2). Cell lines were grown and expanded in DMEM medium (Gibco, Life Technologies) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Life Technologies), 1% PenStrep antibiotic (Invitrogen, Life Technologies) at 37°C and 5% CO_2 atmosphere.

Preparation of RNA samples and microarray analysis

RNA was harvested from the 50 tumor and 30 benign/normal breast tissues using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Harvested RNA was assessed for degradation as well as quantity and purity, then aliquoted and stored at −80°C.

For microarray studies, we carried out analysis for each molecular subtypes of breast cancer (Luminal A, $n=4$, Luminal B, $n=4$, Her2+, $n=2$, triple negative, $n=2$) compared to matching controls obtained from the same patients in triplicate using the Human Genome U133 plus 2.0 GeneChip oligonucleotide arrays (Affymetrix). The chip contained pairs of matched/mismatched 25-mer oligonucleotide probes for over 47,000 transcripts of known genes.

Quantitative RT-PCR analysis (RT-qPCR)

RT-qPCR was used to detect *BRIP1* mRNA in breast cancer cell lines as well as tumor tissue samples (42 tumor and 21 normal/benign breast tissue samples). cDNA synthesis was carried out using High-Capacity cDNA Reverse Transcription Kit (Applied BioSystems) according to the manufacturer's protocol. Briefly, 200 ng of total RNA was reverse transcribed using the following program: 25°C for 10 min, 37°C for 120 min and 85°C for 5 min, followed by incubation at 4°C.

RT-qPCR was performed using TaqMan reagents according to the manufacturer's protocol. Briefly, 10 µL of TaqMan Expression Master Mix were added to 100 ng of cDNA and 1 µL of TaqMan Gene Expression Assay containing primers and probes (*BRIP1* and *GAPDH*, Thermo Scientific Fisher), appropriate volume of water was added to bring the total volume to 20 µL. RT-qPCR was then carried out in the ABI 7500 Fast real-time PCR machine (Applied Biosystems) using the following program: UNG incubation at 50°C for 2 min, polymerase activation at 95°C for 20 s, followed by 40 PCR cycles of denaturation at 95°C for 30 s and annealing/elongation at 60°C for 30 s. All reactions were performed in triplicates and the relative expression levels of *BRIP1* were calculated by normalizing the cycle threshold values of *BRIP1* with those of *GAPDH*. Relative expression of *BRIP1* was analyzed in each molecular subtype, using the comparative CT method.

Western blot

Western blot was performed in breast cancer cell lines and tissue samples (42 tumor samples and 21 benign samples).

Samples were lysed in RIPA buffer supplemented with 0.1 mmol/L of a protease inhibitor cocktail, sodium orthovanadate and phenylmethylsulfonylfluoride (PMSF) (Santa Cruz Biotechnology). The lysate was incubated on ice for 30 min and vortexed briefly every 10 min, then centrifuged at 17,000 ***g*** for 15 min to collect the proteins. The final protein concentration in the supernatant was determined using the Bradford Protein Assay Reagent (Pierce).

Equal amounts of protein (~40 µg) were boiled for 5 min in an equal volume of reducing buffer, resolved on 8% polyacrylamide gels and electroblotted onto nitrocellulose membranes. Membranes were probed with an anti-BRIP1 (1:500 dilution, Abcam: abID#151509) and anti-β-actin primary antibodies (1:200 dilution, Santa Cruz Biotechnology), followed by a goat anti-rabbit IgG-HRP (1:10,000 dilution, Santa Cruz Biotechnology) secondary antibody. Immunoreactivity was detected using chemiluminescence as recommended by the manufacturer (Pierce Biotechnology).

In order to obtain a relative quantification of gene expressions, images acquired from Western blotting were analyzed using ImageJ software. The intensity of the BRIP1 bands relative to the β-actin bands were used to calculate a relative expression of this gene in each cell line.

PCR amplification and sequencing

Breast tissue samples (50 tumors and 30 normal/benign tissues) were collected from patients and genomic DNA was isolated using the QiAMP DNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The primers were designed by Primer3 software and

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tested for specificity using public databases (Human Genome NCBI and BLAST analysis) (3). The primers used were also described in previous studies (4, 5) and obtained from Metabion International AG (Steinkirchen, Germany).

The full coding sequence of the *BRIP1* gene was amplified using primers specific for each of the 20 exons (Table 1). Amplification included an initial denaturation at 95°C for 7 min, followed by 35 cycles composed of denaturation at 95°C for 30 s, annealing at temperature ranging from 50 to 62°C depending on each primer's melting temperature (Table 1) for 30 s and an elongation at 72°C for 30 s. Samples were ultimately incubated for 10 min at 72°C for a final extension. The PCR product from each exon was resolved by using 1.5% agarose gel electrophoresis.

To determine whether *BRIP1* was mutated in breast cancer, PCR products were sequenced using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The conditions of the sequencing reaction included 25 cycles at 96°C (10 s), 60°C (5 s), 60°C (4 min) and 4°C (holding temperature). Sequencing data analysis was performed using the Chromas Pro version 1.7.7 software, to interpret the sequencing results by comparing the normal sequence of the targeted gene *BRIP1* to the tested sequence.

Statistical analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS, version 23). *T*-test was performed and graphs were plotted using GraphPad Prism Software (version 7.00) to determine fold changes. The significance was attributed to *P* values lower than 5% ($P<0.05$).

Continuity correction chi-square test (50 tumors and 30 normal/benign breast tissues) was performed to analyze any potential associations between the polymorphisms and breast cancer disease.

Kaplan–Meier method was used for survival analysis and significant differences between gene expression and overall survival was compared using the log-rank test. Based on a previous study (6), breast cancer patients were classified into high or low expressing group according to whether the expression of *BRIP1* gene was greater than the median expression of *BRIP1*.

Results

Clinical and pathological characteristics of patients

Fifty female patients with a pathologically confirmed diagnosis of invasive breast cancer were included.

Table 1 Primers and annealing temperatures (T_a) for *BRIP1* coding exons.

Exon	Primer sequence (5′→3′) Forward	Reverse	T_a (°C)	Product size (bp)
BRIP1				
1	CCGGGACTGGTTGATTCCTG	GGACTTCCCTCCGACTTGC	62	305
2	TTCTTTGTAAGGCGTGTCTCAA	CAAATACTCAATGTACTTTATGGGTCA	54	246
3	AACTTCAGATGGAGAGAGACCTTTA	CTGTATTATATTTTCTCAGATCCCAGT	54	241
4	CTGGGTGAACTGGGCTGTAG	GGATTTTTGACCACTCTGTGC	62	250
5	TCTTCTAGAAAAATTGCCTACCTG	GACTACCATGTTCAGCTGTAACTAACT	54	299
6	GGCCTTTGAGAACAACTAGCC	TGGTTTAGAAAATTCCATATCTTCC	54	481
7	TTCCATGTGAGGTTTGATAACG	GCAGTTAATTTGATTTTCCGAAG	54	282
8	GCCTTGTGGCTTTAATGATG	CAACATTTACATCTCCATGAGTAGG	54	349
9	TGAAATATCTTGCCTGCTGTTG	TTTAAATACTCTGGCATAATCAAACA	55	249
10	GTGTGTGTAAGGATGATACTGGTT	TTGCTATATTTAACAATTCTGGGTTA	54	268
11	TTTTCCAATTCCCTCCCAAC	TGCTAGCATCCAAATTAGGCTAT	58	286
12	TTGTATGTAGCTGGGTCATAGGTT	AAAATGCTGGTACTGAGCAAGA	58	295
13	ACTTTGCCCAGCCTCTTCTA	TTACTTGCTGGCACTTCAGG	50	375
14	TTCCATGCCTTTTTCAGG	GCATGCCAATGTTTAAAATGTA	52	382
15	GCCGTAGTCACATTGGCTTA	AATTTATTTTCTTTTCACTCAGGATT	55	261
16	TTTTCAAAATGACAAGAATAAGCA	GGGATCCCTGCAATTAACTTT	58	362
17	TGAAAAACAAAATAAAATCTCTACCC	CCAGTTCCTATGGTTCCAGTT	55	362
18	CTGTCCCACTGGAAAACTGG	CATGTTATGTGTTTTTCACCACAA	54	456
19	GGTTACTTCACTAGAAAAAGCAAGTG	TCAAAGGTAAATGGGAAGAACTTT	54	516
20a	GCAATTATGTTAGCTAGGAGCAGA	TCAAAATCTCTATTTGAAGTGGACTG	55	305
20b	TGAACATCCGCTCTGTTCTG	TGCAATCCTCAGCTTTCACT	55	328
20c	AACAATTCAGATTGCATTTTAGC	GAGTTTAACATAAGCATGATGAC	55	295

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The mean age of all patients was 46.54 (standard deviation (S.D.), ±15.8) years. Majority of patients (84%) did not have any family history of breast cancer or any other cancer. Most of the patients (74%) were pre-menopausal and 62% had less than 50 years old (≤50 years). Axillary lymph nodes were found to be involved in 64% of patients (Table 2).

All patients had invasive ductal carcinoma except for three patients, with invasive lobular carcinoma and nine patients with metaplastic carcinoma. With respect to the hormone receptor, 35 (70%) and 29 (58%) of the patients (50 patients) expressed estrogen and progesterone receptors, respectively. Information regarding Her2/neu status was available for 45 patients and eighteen were Her2 positive. Status for Ki67 proliferative index was available for 20 patients, 3 of which had low (<10%) proliferative index, 5 were borderline (10–20%) and the remaining 12 had a high (>20%) proliferative index of Ki67 (Table 2).

Table 2 Clinico-pathological characteristics of patients with breast cancer.

Characteristics	Number of patients	Percentage (%)
Age (years)		
>50	19	38
≤50	31	62
Site of breast lesion		
Left	32	64
Right	18	36
Family history		
Yes	8	16
No	42	84
Menopausal status		
Pre-menopausal	37	74
Post-menopausal	13	26
Histology		
Invasive ductal carcinoma	38	76
Invasive lobular carcinoma	3	6
Metaplastic carcinoma	9	18
Lymph node involvement		
Yes	32	64
No	18	36
Estrogen receptor (ER) status (50 patients)		
ER+	35	79
ER−	10	30
Progesterone receptor (PR) status (50 patients)		
PR+	29	58
PR−	16	42
Her2 receptor status (45 patients)		
Her2+	18	40
Her2−	27	60
Ki67 proliferative index (PI) (20 patients)		
Low (<10%) PI	3	15
Borderline (10–20%) PI	5	25
High (>20%) PI	12	60

Based on their Her2/neu hormone receptor status available for 45 patients, they were categorized based on their molecular subtypes, Luminal A (20), Luminal B (14), Her2+ (5) and triple negative (5). Of the 50 patients, 6 were excluded from the study since their Her2/neu hormone receptor status was not available.

Identification of *BRIP1* as a target in Omani breast cancer patients

To identify the potential genes that underpin the transition of benign to malignant breast tissue, we carried out a microarray gene expression profiling comparison of Omani breast cancer tissue samples from the four molecular subtypes (Luminal A, Luminal B, Her2+ and triple negative) and benign tissues. TAC suite and Ingenuity Pathway Analysis identified genes based on their functional annotations including development, cellular differentiation, proliferation, cytostasis in breast cancer. From all the four groups, *BRIP1* (BRCA1-interacting protein C-terminal helicase1) was identified as a potential BC candidate gene, showing an average of 5-fold overexpression (Fig. 1).

Screening for mutations/SNPs in *BRIP1* gene

Screening for *BRIP1* mutations/deletions by direct sequencing identified four polymorphisms. Sequential analysis of the promoter region, upstream of exon 1, led to the identification of the previously reported polymorphism (c.-141-64G>A) (7). Furthermore, we identified three polymorphisms located within the BRCA1-binding domain; one of them was non-synonymous (c.2755T>C), while the other two were synonymous (c.2637G>A and c.3411T>C). There was no significant association between the two polymorphisms and the presence of breast cancer ($P>0.05$) (Table 3).

Validation of *BRIP1* by RT-qPCR and Western blot analyses

To validate and confirm that *BRIP1* is a potential target, we evaluated mRNA expression of *BRIP1* using RT-qPCR on breast cancer cell lines (MCF-7 and MDA-MB-231) as well as breast tumor tissue samples (42 tumor samples) and compared to the expression in the 21 normal/benign breast tissue samples. *BRIP1* expression was normalized to expression levels of *GAPDH* gene. Results showed, *BRIP1* mRNA levels were 5.2-fold higher in MCF-7 and 1.5-fold higher in MDA-MB-231 ($P<0.05$) (Fig. 2A), analysis in

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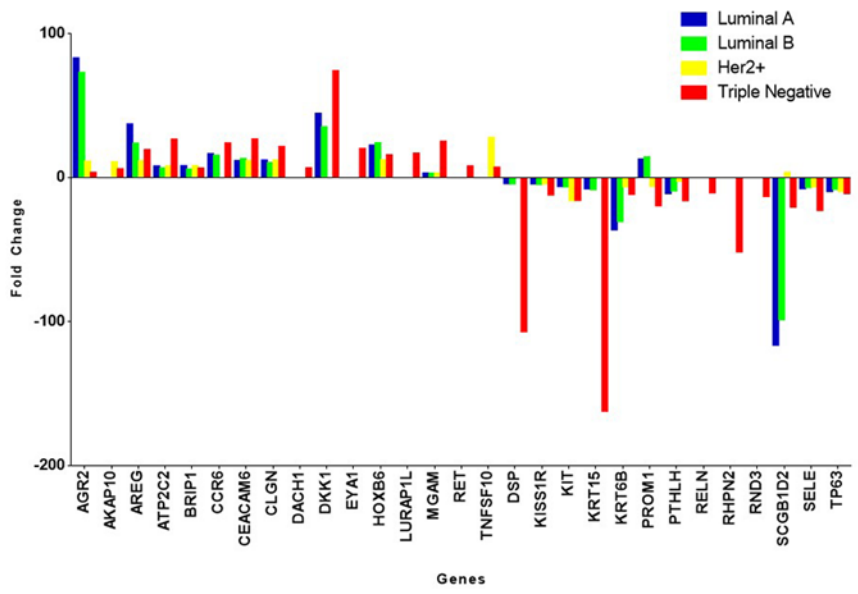

Figure 1
Representative bar graph indicating the expression of other genes including BRIP1 based on the tumor subtype. The blue bar indicates expression of genes in the Luminal A subtype, green indicates gene expression in Luminal B subtype, while yellow and red bars indicate gene expression in the Her2+ and Triple-negative subtypes, respectively.

each molecular subtype showed Luminal A subtype had maximum overexpression of *BRIP1* (6.5-fold) followed by Luminal B (5.2-fold). Both the Her2+ and triple-negative subtype had similar degree of *BRIP1* expression (3.8-fold) as compared to the normal/benign tissue samples ($P<0.01$, Fig. 2B). We also performed, RT-qPCR based on the different grades of the breast tumor in comparison to the normal/benign tissues and analysis showed *BRIP1* expression to increase with increase in grade ($P<0.05$, Fig. 3).

BRIP1 expression was further confirmed for the breast cancer cell lines as well as 42 tumor and 21 normal/benign tissue samples, at the protein levels by Western blotting analysis. Furthermore, MCF-7 displayed a 5.5-fold difference and MDA-MB-231 displayed a 2-fold increase in BRIP1 expression ($P<0.05$, Fig. 4A and B); similar to results obtained in each molecular subtype (Fig. 4). BRIP1 expression was highest in the Luminal A subtype (11.43-fold), followed by Luminal B (4.54-fold), triple-negative (4.86-fold) and Her2+ (5.6-fold) subtypes ($P<0.0001$, Fig. 5A and C). These results were consistent with our RT-qPCR results.

Association of *BRIP1* overexpression with the clinicopathological parameters in breast cancer

BRIP1 expression was analyzed in breast tissue samples by RT-qPCR. The median expression of *BRIP1* obtained by RT-qPCR recorded an 8-fold expression and set as a threshold for overexpression. Patients were then classified into two groups according to their *BRIP1* expression and the overall survival (OS) of patients vs BRIP1 expression was analyzed. A significantly worse overall survival (OS) in patients displaying high *BRIP1* overexpression was obtained ($P<0.05$, Fig. 6). Survival analysis was also performed for the Luminal A and Luminal B molecular subtypes and the OS of patients displaying high *BRIP1* expression was significantly worse when compared to patients with low *BRIP1* expression in both the Luminal A and Luminal B subtypes ($P<0.05$, Fig. 7A and B). We could not perform analysis for Her2+ and triple-negative subtypes due to the low number of patients.

Table 3 Identified variants in *BRIP1* among breast cancer patients.

Exon	Region	rs#	Nucleotide change[a]	Effect on protein	Minor allele frequency in patients	Frequency in controls	*P* value
Polymorphisms							
1	5′-UTR	rs2048718	c.-141-64G>A	–	35/50	24/30	0.711
19	*BRCA1* binding domain	rs4986764	c.2755T>C	p.Ser919Pro	40/50	25/30	0.393
19	*BRCA1* binding domain	rs4986765	c.2637G>A	p.Glu879Glu	48/50	29/30	0.879
20	*BRCA1* binding domain	rs4986763	c.3411C>T	p.Tyr1137Tyr	49/50	30/30	0.435

[a]Numbering based on RefSeq NM_032043 (for all the variants within the coding region). For exonic variants, numbering starts at codon 1.

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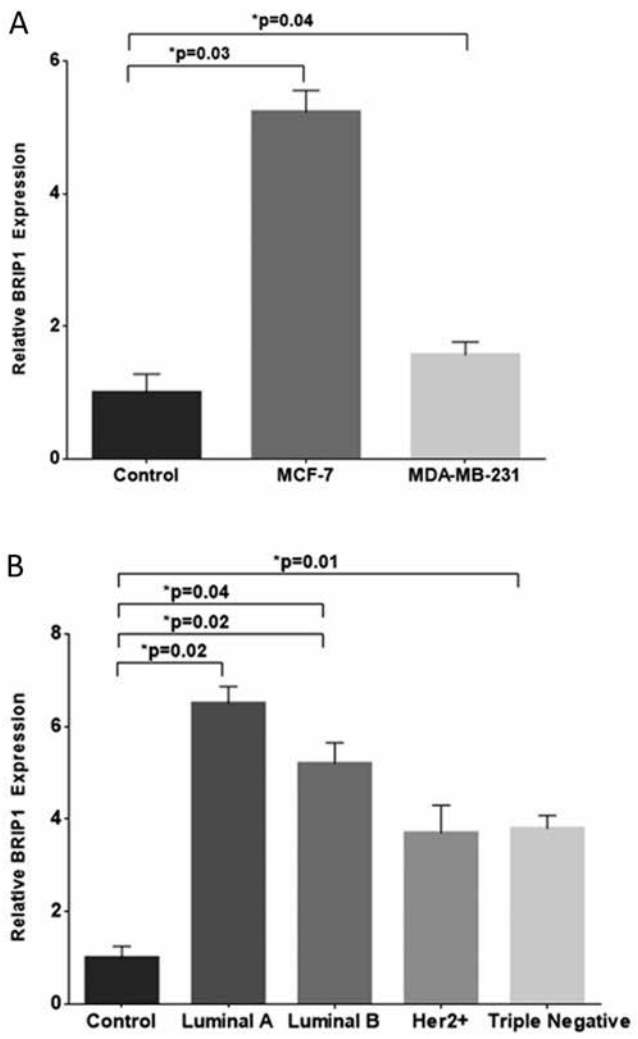

Figure 2
Validation of *BRIP1* expression by quantitative RT-PCR in breast cancer cell lines and breast tissue samples. RT-qPCR was carried out on cell lines and breast tissue samples. (A) MCF-7 had maximum *BRIP1* expression with 5.2-fold, followed by a 1.5-fold expression for MDA-MB-231 in comparison to normal/benign breast tissue samples ($P<0.05$). (B) Analysis in each molecular subtype showed Luminal A subtype had maximum overexpression of *BRIP1* (6.5-fold) followed by Luminal B (5.2-fold). Both the Her2+ and Triple-negative subtype had similar degree of *BRIP1* expression (3.8-fold) as compared to the normal/benign tissue samples ($P<0.01$).

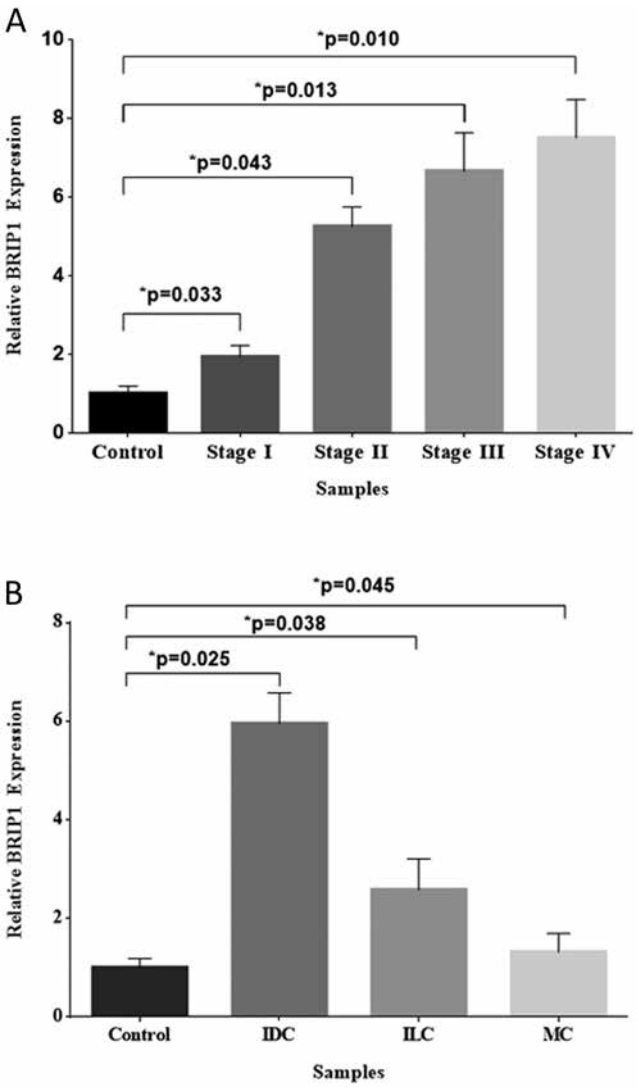

Figure 3
(A) Analysis of *BRIP1* expression in the different stages (1–4) of breast cancer by quantitative RT-PCR in breast tissue samples. RT-qPCR was carried out on breast tissue samples and expression was compared to the normal/benign breast tissue samples. As the stage of the tumor increased, *BRIP1* expression also increased in comparison to normal/benign breast tissue samples ($P<0.05$). (B) Analysis of *BRIP1* expression based on the histology of breast cancer by quantitative RT-PCR in breast tissue samples. RT-qPCR was carried out on breast tissue samples and expression was compared to the normal/benign breast tissue samples. When divided by invasive ductal, invasive lobular and metaplastic carcinoma, *BRIP1* was highly expressed in the invasive ductal followed by the invasive lobular and metaplastic carcinoma in comparison to normal/benign breast tissue samples ($P<0.05$).

Discussion

In this study, we used microarray gene expression profiling to identify differentially expressed genes with a key role in the pathogenesis of breast cancer. Initial data analysis identified potential up/downregulated genes that might be associated with the specific signaling pathways promoting the transition from benign/normal breast tissue to malignant tumor (Fig. 1, q-value <0.01).

As indicated in Table 2, more than half of the patients were below the age of 50 years and presented with invasive phenotype and more lymph node involvement. Similarly, previous data showed that women affected with breast cancer at a younger age tend to have an advanced invasive pathological type, higher tumor grade and higher rates of

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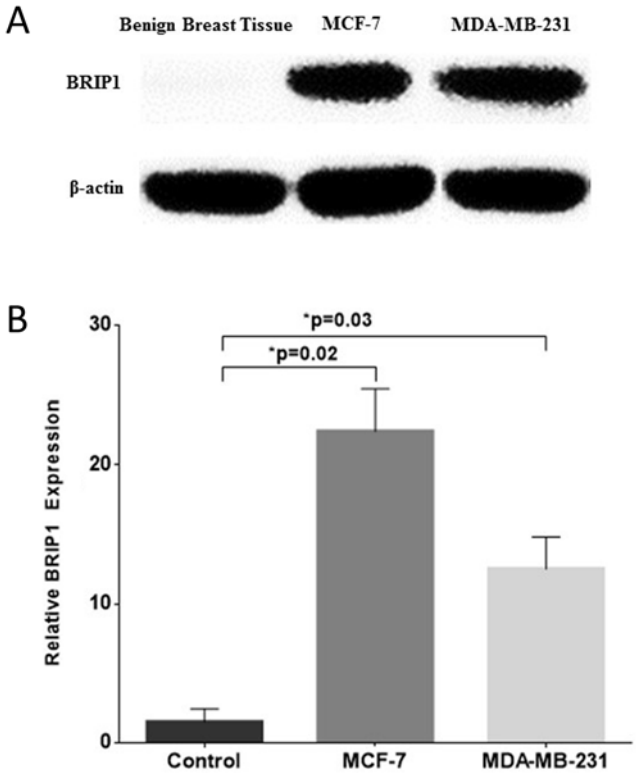

Figure 4
Validation of BRIP1 by Western blot in breast cancer cell lines.
(A) Immunoblotting analysis showed elevated BRIP1 expression in breast cancer cell lines. β-Actin was used as a control for loading.
(B) Quantification showed that MCF-7 displayed a 14.9-fold increase and MDA-MB-231 displayed an 8.2-fold increase in BRIP1 expression as compared to the benign/normal breast tissue ($P<0.05$).

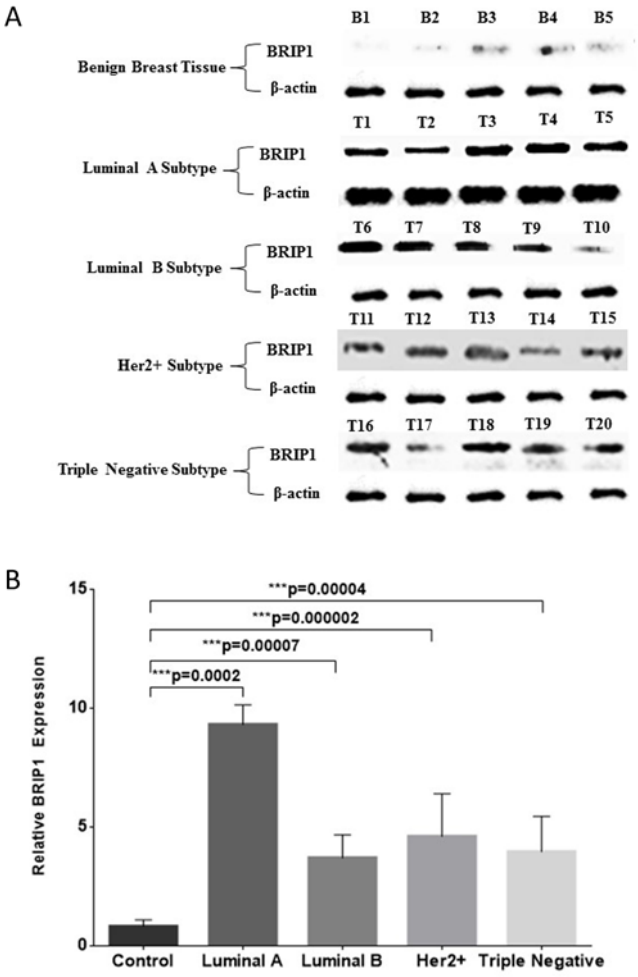

Figure 5
Validation of BRIP1 by Western blot in breast tissue samples.
(A) Validation was carried out for all the 42 tumor and 21 normal/benign tissue samples, at the protein levels by Western blotting analysis. For the benign samples, the five wells correspond to the 5 benign tissue samples (B1–B5). For the Luminal A subtype, the five wells correspond to the 5 tumor tissue samples belonging to the Luminal A subtype (T1–T5); for the Luminal B subtype, the five wells correspond to the 5 tumor tissue samples belonging to the Luminal B subtype (T6–T10). For the Her2+ subtype, the five wells correspond to the 5 tumor tissue samples belonging to the Her2+ subtype (T11–T15) and for the triple-negative subtype, the five wells correspond to the 5 tumor tissue samples belonging to the triple-negative subtype (T16–T20). The same description has been added into the figure legend. (B) Quantification BRIP1 expression was the highest in Luminal A subtype (11.43-fold), followed by Luminal B (4.54-fold), triple-negative (4.86-fold) and Her2+ (5.6-fold) subtypes ($P<0.0001$).

lymph node positivity (8, 9, 10). The majority of patients (84%) lacked a family history and failed the routine *BRCA1/2* clinical test, indicating the lack of inherited *BRCA1/2* mutations, and therefore, unlikely to play a key role in the development of sporadic breast tumors (11, 12). However, these sporadic cancers could possibly have the *BRCA*ness phenotype (13, 14), which prompted us to look for other genes with a role in the onset of breast cancer either independently or in association with *BRCA1/2*, such as the role played by genetic modifiers (15). One potential candidate interacting with *BRCA1/2* to regulate DNA repair and cell cycle was identified as *BRIP1* gene, displaying 5-fold expression in the breast tumors as compared to the normal/benign tissue (Fig. 1).

Validation analysis using RT-qPCR (Fig. 2B, $P<0.01$) and Western blot analyses (Fig. 5, $P<0.0001$) carried out on tumor as well as benign tissue samples showed an overexpression of *BRIP1* in tumor samples as compared to benign samples; a characteristic feature of oncogenes. Results were consistent with the microarray analysis.

Although *BRIP1* is often described as a tumor suppressor gene, our present data are rather compatible with an oncogenic role, suggesting its dual role in cancer as a TSG but also as an oncogene. Interestingly, a study on the TSG *TP53* showed that an overexpression at the mRNA levels is independent of the presence or absence of mutations. This was considered as an early event, not dependent on

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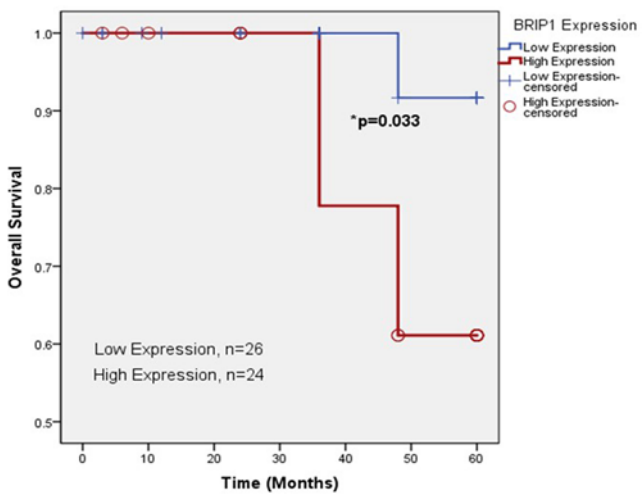

Figure 6
OS in patients expressing *BRIP1*. Kaplan–Meier estimates of 5-year OS according to *BRIP1* expression. High expression of *BRIP1* significantly correlated with poor OS and prognosis ($P<0.05$).

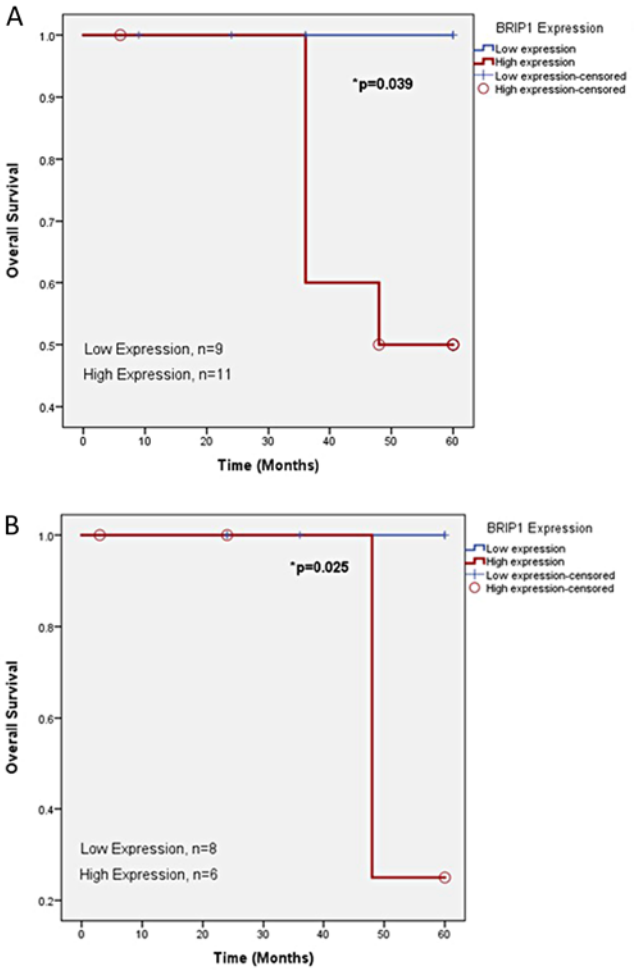

Figure 7
OS in patients belonging to the Luminal A and Luminal B subtypes expressing *BRIP1*. (A) Kaplan–Meier estimates of 5-year OS according to *BRIP1* expression. High expression of *BRIP1* in patients belonging to the Luminal A subtype significantly correlated with poor OS and prognosis ($P<0.05$). (B) Kaplan–Meier estimates of 5-year OS according to *BRIP1* expression. High expression of *BRIP1* in patients belonging to the Luminal B subtype significantly correlated with poor OS and prognosis ($P<0.05$).

the stage of colon cancer and suggesting that p53, tumor suppressor role may turn to an oncogenic role (16, 17, 18).

RT-qPCR (Fig. 2A, $P<0.05$) and Western blot analyses (Fig. 4, $P<0.05$) in breast cancer cell lines (MCF-7 and MDA-MB-231) depicted overexpression of *BRIP1* in MCF-7 followed by MDA-MB-231; results similar to a study where *BRIP1* was expressed in MCF-7 and absent in MDA-MB-231 (20). Furthermore, *BRIP1* knockdown by siRNA in cells with amplification of *BRIP1* (HCC-1954 and MCF-7) showed reduced cellular growth and proliferation as compared to cells lacking *BRIP1* amplification (MDA-MB-231 and MCF-10A) (20). Moreover, along with MCF-7, another breast cancer cell line, BT474 also displayed *BRIP1* amplification (21). Interestingly, another study showed overexpression of *BRIP1* to enhance malignancy of breast cancer cells and its knockdown reduced bone metastasis (22), which is in support of *BRIP1* as an oncogene.

To further explain the role of *BRIP1* in breast cancer, immunohistochemistry was performed on formalin-fixed paraffin-embedded tissues from breast cancer. BRIP1 expression was higher in breast cancer as compared to normal breast (data not shown), in accordance with our RT-qPCR, where *BRIP1* expression increased with increase in grade of breast cancer (Fig. 3, $P<0.05$). Similarly, a study on 101 invasive breast cancers showed that BRIP1 expression was higher in grade 3 carcinomas as compared to grades 1 and 2 (19).

BRIP1 overexpression results in a gain of function, and it is present in a large complex of transcriptional regulators, co-regulators and chromatin modifiers (20). *BRIP1* regulates the function of several metastatic promoting genes including *DUSP1*, *FHL1*, *MMP1* and *CXCR4*. The latter two genes regulate tissue remodeling, tumor progression and invasion (22). Along with this study, two other studies have indicated *BRIP1* as a pro-metastatic gene (23) involved in the regulation of prostate cancer progression (24). Furthermore, *BRIP1* was found to be a direct target of the tumor suppressor micro-RNA Let-7, a regulator and predictor of BC metastasis (23). *BRIP1* cloned to a GAL4 DNA-binding domain showed strong transcriptional repression independent of its ability to bind *BRCA1* (S900A) or its helicase activity (K52R) (20), thus indicating its amplification in sporadic breast cancer.

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Based on several studies and with the identification of several mutations (truncated, germline and missense), *BRIP1* was identified as a breast cancer predisposing gene, and several studies have revealed the association of *BRIP1* mutations with breast cancer susceptibility (4, 5, 7, 25, 26). Although the pathogenicity of *BRIP1* mutations has not been convincingly proved so far, it can possibly help in understanding the non-*BRCA1/2* breast cancer cases (27).

In this study, tumors from patients who lack functional *BRCA1/2* mutations were examined for the presence of aberrations by screening the whole coding area of the *BRIP1* gene. We were unable to identify any significant aberration except previously identified polymorphisms (c.2755C>T, c.-141-64G>A, c.2637G>A and c.3411T>C) (Table 3).

The first common polymorphism identified in our study was located in the 5′-UTR region (rs2048718) (Table 3) and several earlier studies on this variation were carried out in small as well as large cohorts of breast cancer cases and revealed no association with breast cancer (7, 25, 28, 29, 30, 31). Similarly, in this study, no association was established between the polymorphism (rs2048718) and breast cancer (Table 3, $P>0.05$). Although it is generally more frequent in the control series, this 5′-UTR variant affects however cell proliferation and cell growth (32). However, since it is located in the regulatory site, H3K27ac region, which is associated with active enhancer, and it might alter gene expression under the influence of environmental factors.

We also identified a non-synonymous polymorphism (rs4986764) (Table 3) in exon 19 and lacked significant association with breast cancer (Table 3, $P>0.05$). Similar to our results, studies indicated that the common variant, rs4986764 (p.Ser919Pro), found in the *BRCA1*-binding domain (33), lack significant associations with breast cancer risk (4, 7, 28, 29, 33, 34, 35, 36, 37). Curiously, incomplete segregation patterns were identified for susceptibility alleles that can be associated with breast cancer risk (38), suggesting that further analysis of the identified variants, rs4986764 and rs2048718 are needed.

We further identified two synonymous polymorphisms rs4986765 and rs4986763 in exon 19 and 20, respectively (Table 3). These polymorphisms are characterized by the tendency to decrease the binding of the splicing factor, SC35, which is required for the formation of the earliest ATP-dependent splicing complex. This interacts with the spliceosomal components bound to both the 5′- and 3′-splice sites of *BRIP1* during spliceosome assembly (7). Hence, the two silent mutations (rs4986765, rs4986763) could be involved in the repression or promotion of splicing or alternative splicing of the *BRIP1* gene (7). Although these alterations have been investigated in several breast cancer case–control studies to determine their association with breast cancer susceptibility, no studies have been performed so far to demonstrate a significant association of these silent variants with breast cancer (7, 29, 33, 34, 35, 36, 39). This study showed no significant association between the two polymorphisms and the presence of breast cancer (Table 3, $P>0.05$).

In the present cohort, 24 patients overexpressed *BRIP1*, while 26 lacked this expression. We further found overexpression of *BRIP1* to be associated with poor OS and poor prognosis ($P<0.05$, Fig. 6). We also analyzed the OS in the Luminal A and B molecular subtypes and found *BRIP1* overexpression associated with poor OS in patients belonging to both Luminal A and B subtypes ($P<0.05$, Fig. 7A and B). Our data are similar to those reported on colorectal cancer, where *BRIP1* overexpression correlated with poor recurrence-free survival (40). Also, elevated expression of *BRIP1* was observed in high-grade breast tumors correlated with an unfavorable outcome (19). Due to small number of patients in the Her2+ and triple-negative subtypes, survival analysis could not be performed and needs further evaluation.

Several studies focused on developing suitable inhibitors targeting *BRIP1* to guide the design of appropriate therapeutic strategies against cancer. Small molecules targeting *BRIP1* signaling pathways such as Werner syndrome (WRN) helicase inhibitor have been developed (41, 42, 43). In a recent study, high *BRIP1* expression was associated with poor responsiveness of 5-FU in colorectal cancer (40). In gastric tumors, 5-FU reduced *BRIP1* expression and increased sensitivity to oxaliplatin in gastric tumors (44), suggesting a combination of fluoropyrimidine and platinum agents for the treatment of gastric carcinomas (45). Furthermore, based on *BRIP1's* interaction with BLM, BLM helicase inhibitors could promote sister chromatid exchange (46).

PARP1 inhibitors involved in repairing single-strand breaks and sensitizing *BRIP1*-induced tumors are additional approaches being introduced (27). Moreover, cells lacking *BRIP1* are sensitive to treatment with cisplatin (47). *BRIP1* is involved in repairing DNA inter-strand crosslinks and plays a role in G4-DNA, thus indicating that *BRIP1*-induced tumors is sensitive to telomestatin, a G4-DNA ligand regulating the stabilization of G4-DNA structures (27). These strategies highlight the importance of understanding the underlying mechanisms of *BRIP1* in cancer to establish appropriate and efficient therapeutic

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strategies. Furthermore, enzymes (S990A) targeting *BRIP1* signaling pathways particularly involved in over-reactivity of helicases such as Fe-S domain can pave the way toward the design of useful strategies for cancer treatment (27).

Conclusion

The prevailing scenario of breast cancer disease in this region of the Middle East (Oman) seems to be of sporadic nature, with the absence of the family history in the majority of cases. One of the limitations of this study was the lack of information about the *BRCA1/2* status of the patients, due to ethical regulations and hence those patients who could be carriers of rare *BRCA1/2* variants were not known. In addition, social stigma associated with the results after the test is another reason to refrain patients from taking the test (48). Furthermore, this test is not cost-effective and hence, a proportion of patients are unable to afford it. This situation may change as the technology and awareness becomes more widespread.

Although small sample size was a limitation in this study, we have demonstrated that *BRIP1* gene expression changes in the molecular subtype of breast cancer in Omani patients. Although *BRIP1* is a TSG, data presented in this study, indicates *BRIP1* to be an oncogene in sporadic cases. Overexpression of certain tumor suppressors including *BRIP1*, can lead to genomic instability in cellular processing involved in genome integrity maintenance and contribute to the onset and progression of breast malignancy. The present data suggest a putative role of *BRIP1* in the onset and progression in breast cancer and the possibility to use this gene as a biomarker. In addition, the small-sample size and lack of patients belonging to the Her2+ and triple-negative subtypes could over-estimate the magnitude of an association and hence, future work will involve a larger cohort to establish a reliable association as well as understand the underlying mechanisms. This can also help to design targeted therapeutic strategies for breast cancer. Furthermore, studies are needed to evaluate the significance of *BRIP1* as a predictive and prognostic factor in breast cancer as well as other cancers.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported and funded by 'His Majesty Research Trust Fund', SR/MED/BIOC/14/01.

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Received in final form 11 October 2017
Accepted 14 November 2017
Accepted Preprint published online 14 November 2017