Mutation Analysis of **BRCA1, BRCA2, PALB2** and **BRD7** in a Hospital-Based Series of German Patients with Triple-Negative Breast Cancer

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Abstract

Triple-negative breast cancer (TNBC) is an aggressive form of breast carcinoma with a poor prognosis. Recent evidence suggests that some patients with TNBC harbour germ-line mutations in DNA repair genes which may render their tumours susceptible to novel therapies such as treatment with PARP inhibitors. In the present study, we have investigated a hospital-based series of 40 German patients with TNBC for the presence of germ-line mutations in **BRCA1**, **BRCA2**, **PALB2**, and **BRD7** genes. Microfluidic array PCR and next-generation sequencing was used for **BRCA1** and **BRCA2** analysis while conventional high-resolution melting and Sanger sequencing was applied to study the coding regions of **PALB2** and **BRD7**, respectively. Truncating mutations in **BRCA1** were found in six patients, and truncating mutations in **BRCA2** and **PALB2** were detected in one patient each, whereas no truncating mutation was identified in **BRD7**. One patient was a double heterozygote for the **PALB2** mutation, c.758insT, and a **BRCA1** mutation, c.927delA. Our results confirm in a hospital-based setting that a substantial proportion of German TNBC patients (17.5%) harbour germ-line mutations in genes involved in homology-directed DNA repair, with a preponderance of **BRCA1** mutations. Triple-negative breast cancer should be considered as an additional criterion for future genetic counselling and diagnostic sequencing.


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Competing Interests: ML is employed by Fluidigm Corporation. For this study Target-specific primers were designed by Fluidigm Corp using Fluidigm primer service program. A Fluidigm Access Array was used in these studies. There are no further patents, products in development or marketed products to declare. This does not alter the authors’ adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors.

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Introduction

Triple-negative breast cancers (TNBCs) account for about 15% of all invasive breast cancers and are defined as tumours that lack expression of estrogen receptor (ER) and progesterone receptor (PR), and do not show overexpression of HER2/neu [1]. TNBCs are usually high-grade, invasive ductal carcinomas and have been linked with a worse prognosis [1,2]. There is some overlap with a basal-like pattern of gene expression [2,3]. Women with a breast cancer family history experience a significantly increased risk of triple-negative breast cancer [4]. Importantly, carriers of mutations in the breast cancer susceptibility gene 1, **BRCA1**, frequently have basal-like and/or triple-negative breast cancers [5].

Triple-negative cancers which harbour a dysfunctional **BRCA1** pathway may be sensitive to platinum-based chemotherapy [6] and to inhibitors of the poly(ADP-ribose)-polymerase that selectively target cells deficient in homologous recombination DNA repair [7,8]. Recent studies have indicated that germ-line **BRCA1** mutations might be overrepresented in patients with TNBC, in particular those with an early onset of the disease [9–11]. Deleterious mutations of the second breast cancer susceptibility gene, **BRCA2**, have also been reported to occur at a high frequency in German patients with triple-negative breast cancers [12].

The **BRCA1** protein is involved in homologous recombination-mediated DNA repair through an interaction with **BRCA2** that is mediated by **PALB2**, the partner and localizer of **BRCA2** [13,14]. **PALB2** bridges the **BRCA1** and **BRCA2** proteins and regulates their function in the DNA damage response [14]. **PALB2** mutations have been associated with breast cancer in several studies [15–19]. The **BRCA1** protein is also involved in the transcriptional regulation of the estrogen receptor alpha (**ESR1**) through an interaction with bromodomain-containing protein 7 (**BRD7**), a subunit of the SWI/SNF chromatin remodelling complex. **BRD7** is required for the recruitment of **BRCA1** to the **ESR1** promoter [20]. **BRD7** also interacts with the tumour suppressor p53 and is required for efficient transcription of a subset of p53 target genes [21,22]. **BRD7** is frequently deleted in human breast tumours harbouring wildtype p53 [21] but the potential role of **BRD7** germ-line mutations in breast cancer has not yet been fully elucidated.

In the present study, we scanned the whole coding regions of **BRCA1**, **BRCA2**, **PALB2** and **BRD7** in order to investigate the relative contributions of germ-line mutations in these genes to...
Patients and Methods

Patients

For the present study we ascertained 40 patients who were diagnosed with triple-negative breast cancer during the years 2009–2011 at the Clinics of Obstetrics and Gynecology at Hannover Medical School. Medical records were reviewed with the following information captured on a case report form: demographics (age, date of birth, ethnicity), personal history of cancer, age of diagnosis, recurrence, current status, family history from the time of the patient’s diagnosis. Median age at onset was 52 years (range 22–81 years). 35 of the 40 patients were of German descent, the others were Polish, Tunisian, Korean, Iranian or Filipino. 12 of the 40 patients reported a first-degree family history of breast cancers, two of them also with a first-degree family history of ovarian cancer. The expression of ERα, PR and HER2/ neu was assessed using mAb SP1 (ERα), mAb 1E2 (PR), mAb 4B5 (Her2), mAbs XM26 and LL002 (CK5/14), and mAb 2-1E1/EGFR. Antigen was retrieved by automatically pouring retrieval solution (Ventana) onto sections with subsequent heat treatment. After quenching of endogenous peroxidase activity by immersion in 3% H2O2 for 10 min, tissue sections were incubated with primary antibody at room temperature followed by staining using the UltraView kit (Ventana). All cases showing less than 1% tumor cells expressing ERα or PR and all cases showing an HER2 score of less than 2 were considered negative. For CK5/14 and EGFR a semiquantitative score (0 no expression; 1 weak expression; 2 moderate expression and 3 strong expression) as well as the relative percentage of positive tumor cells was calculated.

Informed written consent was obtained from each patient, and the study was approved by the Institutional Review Board at Hannover Medical School (Ethics commission vote No. 762/10). For each patient, genomic DNA was isolated from peripheral white blood cells using standard phenol-chloroform extraction.

BRCA1 and BRCA2 Analysis

Target-specific primers were designed by Fluidigm Corp (San Francisco) using Fluidigm primer service program with the following recommendations: Tm range of 59–61°C, max of homopolymer is 3 and GC% less than 65%. Common sequence tags (CS1 and CS2) were added to forward and reverse primers for Access Array amplicon tagging experiments. 77 primer pairs were designed and validated to cover all exons except exon 22 (74 bp) in BRCA1 (Figure S1). The exons of BRCA1 and BRCA2 were then amplified from triple-negative breast cancer patients to receive 40 pools of 77 amplicons. For this purpose, each genomic DNA sample was normalised to a concentration of ~50 ng/ml and loaded onto an Access Array (Fluidigm, San Francisco), a microfluidic array in which a PCR was performed with nested primer pairs. Each primary primer pair contained the template-specific sequence and a tag sequence. Each secondary primer pair with sample contained the anti-tag sequence, a sample-specific unique barcode, and the 454 adaptor sequence. PCR products were harvested from each sample and were checked on an agarose gel to confirm uniformity of the amplicon coverage (distribution within 2-fold). PCR products were subsequently pooled using 1 µl per sample and purified, the library was subjected to emulsion PCR and the products were pyrosequenced with a GS FLX 454 system (Roche, Basel).

Sequencing data were analysed with NextGENe 2nd Generation Sequencing Software v.2.2.1 (SoftGenetics, Philadelphia, U.S.A.). In brief, raw data were converted to FASTA files and were aligned to BRCA1 and BRCA2 gbk files from the human reference sequences (GRCh37.p5 Primary Assembly, NC_000017.10 for BRCA1 and NC_000013.10 for BRCA2; http://www.ncbi.nlm.nih.gov/genbank/). Only reads over 25 bases were converted, and reads were rejected if they contained more than 3 uncalled bases. Alignment was performed with a required matching of over 85% within more than 50 bases. This yielded an average of 2.35 million converted reads per sample (range 1.05–3.43×106), and about 95% of the reads could be matched. The average read length per sample was 487 (range 481–491) bases, and the average coverage per sample was 74-fold (range 33–110 fold). The average coverage per exon was above 30-fold (30–240 fold) except for three amplicons (exons 5, 15, and 21 of BRCA1) that were covered less than 20-fold; these three exons and the missing exon 22 of BRCA1 were manually resequenced using BigDye Terminator Cycle sequencing (Applied Biosystems) with exon-flanking intronic primer pairs. For the others, mutation filters were set to exclude mutations with a percentage less than 10% or less than 3 counts, and to exclude homopolymer indels with a forward/reverse balance less than 0.1. Two regions were further inspected manually in each sample as they were largely represented by only one sequenced strand. All identified mutations, apart from common polymorphisms or known synonymous variants, were finally validated by conventional Sanger sequencing using BigDye chemistry and a 3100 Avant Genetic Analyser (Applied Biosystems, Darmstadt).

PALB2 Analysis

All exons of PALB2 were scanned for mutations by high-resolution melting (HRM) analysis as previously described [17]. In brief, PCR amplifications were set up in the presence of the EvaGreen dye (BioBudget, Krefeld, Germany), and high-resolution melting analysis was performed on the Rotor-Gene 6000 real-time PCR machine (Corbett Research, Mortlake, Australia). Melting profiles were evaluated using the Melt Curve Analysis tool of the Rotor-Gene 6000 Series Software Version 1.7. All samples with suspicious melting behaviour were then subjected to direct sequencing to identify the underlying substitution using BigDye chemistry and a 3100 Avant Genetic Analyser (Applied Biosystems, Darmstadt).

BRD7 Analysis

To study genetic variants in the BRD7 coding region, DNA samples were analysed by conventional Sanger sequencing. Primer pairs were designed to amplify each of the 17 coding exons (exons 2–18 of BRD7) including their flanking intron sequences. Primer sequences and PCR conditions are given in Table S1. All PCR products were sequenced using BigDye terminator chemistry v1.1 on a 3100 Avant Genetic Analyser (Applied Biosystems, Darmstadt); the call rate was 100%.

mRNA Analyses

LCLs were established by EBV immortalisation of peripheral white blood cells [23] and were cultured in RPMI 1640 supplemented with 15% heat-inactivated FCS and 1 mM L-glutamine (Biochrom, Berlin, Germany) at 37°C under an atmosphere of 5% CO2. Total mRNA was extracted from cell pellets using a modified guanidium isothiocyanate/phenoil protocol, and 1 µg RNA was reversely transcribed with a First Strand cDNA synthesis kit following the manufacturers instructions (GE Healthcare). One-fifth of the cDNA was included into a
PCR with primers 5'-CTG CAA AGA AGC TGT TGC AC-3'(c7F) and 5'-CTT CCA GTT GTC ATT CCC AG-3' (c11R) spanning the exons 7 through 11 of the BRD7 transcript. RT-PCR products were separated by 2% agarose gel electrophoresis, stained with GelRed and evaluated on a UV transilluminator.

Statistics and Bioinformatics
Genotype frequencies were compared using chi-square analyses, and p<0.05 was considered significant. Fishers exact test was used for numbers <5. Sequence variants were checked for previously published reports in the BIC database (http://research.nhgri.nih.gov/projects/bic/Member/index.shtml), in the PALB2 mutation database (http://www.lovd.nl/PALB2), in the NCBI SNP database (http://www.ncbi.nlm.nih.gov/snp), or in the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed). Exonic sequence variants in BRD7 were tested for possible effects on binding sites of splicing factors SF2/ASF, SC35, SRp40 or SRp55 using ESE Finder 3.0 (http://rulai.cshl.edu/tools/ESE).

Results
We investigated a hospital-based series of 40 consecutive patients with triple-negative breast cancer for mutations in the whole coding sequences of BRCA1 and BRCA2 as well as in the two genes PALB2 and BRD7, which encode interaction partners of the BRCA1 protein. Next-generation sequencing of barcoded BRCA1 and BRCA2 amplicon pools revealed five truncating mutations of BRCA1 in six patients (6/40, 15%) and one truncating mutation of BRCA2 in one patient (1/40, 2.5%) (Table 1). All six mutations create premature stop codons upstream of the penultimate exon and were considered pathogenic. Three of the BRCA1 mutations were known breast cancer-associated mutations that were recorded in the Breast Cancer Information Core (BIC) database. Two of the remaining three frame-shift mutations, c.843_846del4 in BRCA1 and c.5266dupC in BRCA2, were not included in the BIC database but had recently been reported to the NCBI SNP database as rs80357792 and rs80359500, respectively. We did not find a reference for BRCA1 mutation c.927delA indicating that this could be a private mutation (Figure 1).

Some clinical features of the TNBC patients with BRCA1 or BRCA2 mutations are summarized in Table 1. Two of the seven carriers had bilateral breast cancer. Three of the seven carriers had been diagnosed below age 50, compared with 15 out of 33 non-carriers (p = 0.9). Five of the seven carriers had a first-degree family history of either breast or ovarian cancer, compared with nine out of 33 non-carriers (p = 0.05). Six of the seven carriers showed ductal histology, while one BRCA1 mutation carrier had a medullary carcinoma. All seven carriers were of German descent though the carrier of the BRCA1 c.843_846del4 mutation had a Polish background. Mutation carriers were still alive at 1–3 years following standard taxane-based chemotherapy, except for one BRCA1 c.5266dupC carrier who died at one year after diagnosis.

We furthermore scanned the whole coding region of PALB2 in all TNBC patients using high-resolution melting analysis [17]. Apart from known polymorphisms, one sample was identified with an aberrant melting profile and was subsequently sequenced in exon 4 of the PALB2 gene. The patient turned out to be heterozygous for a novel truncating mutation, c.758insT (Figure 1). The insertion of a nucleotide into exon 4 of PALB2 results in a frame-shift and creates a premature termination signal three codons downstream, within the same exon (p.S236X). This PALB2 mutation was identified in the same patient who also carried the novel BRCA1 mutation, c.927delA, and therefore is a double heterozygote for mutations in both genes. The patient was diagnosed with a multifocal invasive ductal carcinoma of stage pT2 and grade 3. Lymph nodes were positive (pN2a, 4/13), but she had no metastases. She also presented with large myomas of the uterus, a small meningioma, visual disturbances due to choroidal neovascularisation, and bipolar disorder. She underwent mastectomy and received chemotherapy with epirubicin and cyclophosphamide which was well tolerated, and she has shown no signs of recurrence three years after diagnosis. One of her three sisters had died from breast cancer by the age of 48 years, and two of three daughters of this sister also had premenopausal breast cancer. No further cancer was known in this family.

We finally considered BRD7 as a plausible candidate gene for TNBC, based on the known interaction of its gene product with BRCA1 in the regulation of estrogen receptor expression. Primer pairs were designed to amplify all 17 coding exons and the flanking intron sequences of BRD7, and the TNBC samples were investigated by conventional Sanger sequencing. We identified two alterations in the coding region both of which were
Table 1. Truncating mutations in BRCA1, BRCA2 and PALB2 among 40 TNBC patients.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>Exon</th>
<th>Consequence</th>
<th>Pathology</th>
<th>BIC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRCA1</td>
<td>c.843_846del4</td>
<td>10</td>
<td>frameshift</td>
<td>Bilateral, medullary/undiff., AD 43 and 53 ys, 1 first-deg OC, 1 second-deg BC.</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>c.927delA</td>
<td>10</td>
<td>frameshift</td>
<td>Multifocal ductal, AD 65 ys, 1 first-deg BC (AD 48 ys) and 2 second-deg BC (AD 35–40 ys). IHC: EGFR pos., CK5/14 neg.</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td>c.4689C&gt;G</td>
<td>15</td>
<td>p.Y1563X</td>
<td>Ductal, AD 49 ys, 1 second-deg BC (AD 63 ys), 1 first-deg DC (AD 47 ys). IHC: CK5/14 pos.</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>c.5153-2delA</td>
<td>IVS18</td>
<td>exon skipping</td>
<td>Ductal, AD 62 ys, 1 first-deg BC. IHC: EGFR neg., CK5/14 weakly pos.</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>c.5266dupC</td>
<td>20</td>
<td>frameshift</td>
<td>Ductal, AD 38 ys, death at 1 year after diagnosis. 1 second-deg BC (AD 30 ys), IHC: CK5/14 pos.</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>c.5266dupC</td>
<td>20</td>
<td>frameshift</td>
<td>Ductal, AD 51 ys, 1 first-deg BC and 9 second-deg BC/OC. IHC: CK5/14 neg.</td>
<td>yes</td>
</tr>
<tr>
<td>BRCA2</td>
<td>c.5238insT</td>
<td>11</td>
<td>frameshift</td>
<td>Ductal, AD 62 ys, 1 second-deg BC. IHC: EGFR weakly pos., CK5/14 weakly pos.</td>
<td>no</td>
</tr>
<tr>
<td>PALB2</td>
<td>c.758insT</td>
<td>4</td>
<td>frameshift</td>
<td>Multifocal ductal, AD 65 ys, 1 first-deg BC (AD 48 ys) and 2 second-deg BC (AD 35–40 ys). IHC: EGFR pos., CK5/14 neg.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

Truncating mutations in BRCA1, BRCA2 and PALB2 among 40 TNBC patients. Mutations were designated according to the improved mutation nomenclature recommended by the Human Genome Variation Society (www.hgvs.org/mutnomen/). AD = age at diagnosis, BC = breast cancer, OC = ovarian cancer, IHC = immunohistochemistry, n.a. = not applicable.


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Synonymous: The known polymorphism c.846C>T in exon 8 (rs1062348) was found at a minor allele frequency of 0.18, which was similar to its reported frequency of 0.24 in the SNP database, and the novel variant c.1861C>T (TCC>TCT; p.S618S) in exon 17 was identified in a single patient. Bioinformatic assessment using ESE Finder 3.0 did not predict alterations in splicing for the rare c.1861C>T polymorphism. However, subsequent inspection of mRNA from two lymphoblastoid cell lines representing both homozygous genotypes did not reveal any alternative splicing in either of both lines (data not shown), indicating that c.846C>T does not disturb BRD7 splicing, at least in lymphoid cells. Altogether, this study did not reveal pathogenic BRD7 alterations and thus the role of germ-line mutations in BRD7 for TNBC, if any, is much less pronounced than the role of BRCA1 mutations in our series of German patients (6/40, p = 0.03).

Discussion

Triple-negative breast cancers (TNBCs) have received much interest at the clinical, biological and epidemiological level due to the aggressive behaviour of the tumour, the poor prognosis and the present inapplicability of therapies with receptor antagonists. Among new therapies that are under investigation [24], the most important one is PARP inhibition, which induces synthetic lethality in tumour cells deficient in homology-directed DNA double-strand break repair such as cells mutated in BRCA1 or BRCA2 [7,8]. BRCA1 mutated tumours are often triple-negative, and the treatment with PARP inhibitors might be promising in at least some TNBC patients [25]. Several recent studies on TNBC have focussed on BRCA1 mutations whereas the prevalence of mutations in BRCA2 or mutations in other genes of the BRCA1 pathway has been less extensively studied. This prompted us to perform a full scanning of the coding region of BRCA1, BRCA2, PALB2 and BRD7 in a consecutive series of German TNBC patients who have visited our hospital over a two-years-period.

BRCA1 and BRCA2 exons were analysed using a Fluidigm Access Array followed by 454 sequencing to save costs and to achieve a high throughput. Combining a microfluidic amplification system with massive parallel sequencing is an effective method for mutation scanning, which has previously been employed for the diagnostics of familial hypercholesterolemia [26] and here was applied to BRCA1 and BRCA2 sequencing. This approach identified a total of seven heterozygotes for truncating mutations in BRCA1 or BRCA2 among the 40 TNBC patients in our series (17.5%) which were all confirmed by Sanger sequencing. These included two patients with BRCA1 mutation c.5266dupC that is common in Eastern Europe and Germany [27]. The rate of 17.5% may be an underestimate as our approach would not have identified large genomic deletions or far intronic mutations. There was no association with earlier age at diagnosis, however more mutation carriers than non-carriers had a first-degree family history of breast or ovarian cancer. Despite this trend towards a positive family history, two of the seven patients would not have been eligible for mutational screening under current guidelines [12] though one of the two would have been detected with expanded criteria including TNBC patients younger than 50 years [28].

BRCA2 mutations were underrepresented in our study compared with BRCA1 mutations which seems to be in contrast with a recent report from another German series of TNBC patients who were also unselected for age at diagnosis and family history [12]. The latter study identified five mutations in BRCA2 and one mutation in BRCA1 in 30 German TNBC patients, whereas we have identified six mutation carriers for BRCA1 and one for BRCA2 in 40 German TNBC patients. Our results are in line with published results from other study populations that consistently find a higher prevalence of mutations in BRCA1 than in BRCA2 [29–33]. Furthermore, BRCA1 mutations were strongly associated with hormone-receptor negative status in previous analyses of
high-risk breast-ovarian cancer families whereas most BRCA2-mutated tumours do not have a TNBC phenotype [34–38]. The biological reason for the different outcomes remains unclear, given that BRCA1 and BRCA2 collaborate in the homology-directed DNA repair pathway, but BRCA1 may exert a particular role in homologous recombination expression. It has also been discussed that BRCA2 mutation carriers may develop TNBC later in life than BRCA1 carriers, and the relatively old age at diagnosis in our BRCA2 mutation carrier may be in line with this hypothesis [33]. Genetic variation at other loci, such as BABAM1 [MERIT40] on chromosome 19q13.1, might further influence the outcome towards a triple-negative phenotype [39].

Similar considerations may apply to PALB2, encoding the partner and localiser of BRCA2 [13]. Mutations in PALB2 are quite rare, and PALB2 was mutated in only one TNBC patient in our series. The PALB2 mutation, c.758insT, was identified in a patient who also carried a BRCA1 mutation, c.927delA, and thus may provide the first example of a case with digenic mutations in these both genes. Double heterozygotes have previously been reported for BRCA1 and BRCA2 mutations and, in one study, appeared to have an earlier onset and a more severe disease than their female relatives carrying a single BRCA1 or BRCA2 mutation [40,41]. While the BRCA1/PALB2 double heterozygous patient described here had no early onset, the multifocal occurrence of the disease and her positive family history may be consistent with a severe predisposition. Given the BRCA1 mutated background, it cannot be decided which of the genes drives the triple-negative phenotype in this patient. Most previous studies have not found evidence that PALB2 mutations were associated with hormone-receptor negative breast tumours [16–18,42], but one study reported that carriers of a Finnish founder mutation in PALB2 had triple negative breast cancer significantly more often than other breast cancer patients [43]. Although the absence of additional carriers in our series argues against an important role of PALB2 mutations in German TNBC patients, a possible link between PALB2 and a triple-negative phenotype still warrants further investigation.

The BRD7 gene was included as an additional candidate gene for TNBC because BRD7 is required for the BRCA1-mediated transcriptional regulation of the estrogen receptor [20]. BRD7 has been described as a tumour suppressor gene that is frequently deleted in human breast tumours harbouring wildtype p53 [21] but no clear pathogenic BRD7 germ-line mutations have been identified in German patients with familial breast cancer, thus far [44]. In our study, there was no pathogenic mutation detected in any of the 40 TNBC patients, excluding a prominent role of BRD7 in the genetic predisposition to this cancer. As our sequencing approach was based on Sanger methodology for BRD7, the absence of mutations was not due to low coverage of certain regions. Although it remains possible that large genomic deletions or far-intronic mutations may exist which would have escaped our detection, there is presently no evidence to implicate BRD7 mutations in the etiology of TNBC.

In summary, this study confirms in a hospital-based setting a substantial proportion of high-penetrance germ-line mutations in German patients with triple-negative breast cancer, with a preponderance of BRCA1 mutations over mutations in BRCA2 or PALB2, and with the exemplification of double heterozygosity for mutations in BRCA1 and PALB2. Triple-negative breast cancer should be considered as an additional criterion for future genetic counselling and diagnostic sequencing.

Supporting Information

Figure S1 Primers for BRCA1 and BRCA2. Barcode primers within BRCA1 and BRCA2, comprised with the adapter sequences for 454, a 10-bp barcode sequences and the common sequence tags. (TIF)

Table S1 Primers for BRD7. Primers sequences for PCR amplification of the coding exons of BRD7 are shown in 5’->3’ direction. Annealing temperatures ranged between 60–66°C. (DOC)

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Author Contributions

Conceived and designed the experiments: FP NB ML TWPS TD. Performed the experiments: FP NB PS FL. Analyzed the data: FP NB PS FL. Contributed reagents/materials/analysis tools: AA PH HC TWPS. Performed the experiments: FP NB PS FL. Contributed reagents/materials/analysis tools: AA PH HC TWPS. Wrote the paper: TD. Revision of the manuscript: TD FP NB ML TWPS TD. Performed the experiments: FP NB PS FL TWPS. Wrote the paper: TD. Revision of the manuscript: TD FP NB ML AA FL PH HC TWPS.

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