Spectrum and frequencies of $BRCA1/2$ mutations in Bulgarian high risk breast cancer patients

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Abstract

Background: About 3885 women are diagnosed with breast cancer and 1285 die from the disease each year in Bulgaria. However no genetic testing to identify the mutations in high-risk families has been provided so far.

Methods: We evaluated 200 Bulgarian women with primary invasive breast cancer and with personal/family history of breast cancer for the presence of unequivocally damaging germline mutations in $BRCA1/2$ using Sanger sequencing.

Results: Of the 200 patients, 39 (19.5 %) carried a disease predisposing mutation, including 28 (14 %) with a $BRCA1$ mutation and 11 (5.5 %) with a $BRCA2$ mutation. At $BRCA1$, 6 different mutations were identified, including 2 frameshifts, 1 nonsense and 1 missense that had been previously reported (c.5030_5033delCTAA, c.5263_5264insC, c.4603G>T, c.181T>G), and 2 frameshifts, which were novel to this study (c.464delA, c.5397_5403delCCCTTG). At $BRCA2$, 7 different frameshift mutations were identified, including 5 previously reported (S851_S854delAGTT, c.946delT, c.5718_5719delCT, c.7910_7914delCCTTTG, c.9098_9099insA) and 2 novel (c.8532_8533delAA, c.9682delA). A $BRCA1$ mutation was found in 18.4 % of women diagnosed with breast cancer at/or under the age of 40 compared to 11.2 % of women diagnosed at a later age; a $BRCA2$ mutation was found in 4 % of women diagnosed at/or under the age of 40 compared to 6.5 % of women diagnosed at a later age. A mutation was present in 26.8 % patients with a positive family history and in 14.4 % of women with a negative family history. The most prevalent mutation observed in 22 patients (11 %) was $BRCA1$ c.5263_5264insC, a known Slavic mutation with founder effect in Eastern European and AJ communities. Other recurrent mutations were $BRCA2$ c.9098-9099insA (2 %), $BRCA1$ c.181T>G (1 %) and $BRCA2$ c.5851_5854delAGTT (1 %). Notably, $BRCA1$ c.5263_5264insC represented 56 % of all mutations identified in this series. Of the 22 patients with $BRCA1$ c.5263_5264insC, 9 were diagnosed with early onset breast cancer, 11 with TNBCs, 4 with bilateral breast cancer, and 6 with both breast and ovarian cancer.

(Continued on next page)
Conclusions: This is the first comprehensive study of the BRCA1/2 mutation spectrum in Bulgaria and will assist the establishment of efficient protocols for genetic testing and individualized risk assessment for Bulgarian breast/ovarian cancer patients and healthy individuals at a high-risk.

Keywords: BRCA1, BRCA2, Breast cancer, Genetic testing, Mutations, Sequencing

Background
Breast cancer (BC) is the second most common cancer in the world and, by far, the most frequent malignant disease among women with an estimated 1.67 million new cases diagnosed in 2012 (25 % of all cancer cases) [1]. Despite the advancement of diagnostic techniques and treatment in the last decade, BC is still the most frequent cause of cancer death in women in less developed regions (324.000 deaths, 14.3 % of total) and the second cause of cancer death in more developed regions (198.000 deaths, 15.4 %) after lung cancer [1].

The etiology of BC is multifactorial and includes both environmental and genetic factors, as well as genetic and epigenetic changes during progression. Up to 5–10 % of all BC cases and 10–15 % of all ovarian cancer (OC) cases are due to germline mutations in one of the two breast cancer susceptibility genes, BRCA1 [MIM#113705] and BRCA2 [MIM#600185] [2–4].

Germline mutations in BRCA1 or BRCA2 explain about 50 % of disease aggregation in severely affected BC and OC families while their prevalence is lower among BC and OC patients unselected for family history or age of diagnosis: BRCA1 mutations are found in <1–7 % cases and BRCA2 mutations in 1-3 % cases [5]. However, as high a proportion as 84 % has been proposed for certain families [6]. Higher prevalence is associated with a family history of BC and/or OC, early onset, male BC or multiple tumours such as bilateral breast cancer (BBC) or BC and OC in the same patient [5]. Furthermore, some histopathologic features such as lack of expression of estrogen, progesterone, and HER2 that define the biologically aggressive and difficult to treat Triple Negative Breast Cancers (TNBC) subtype, have been also attributed to deleterious changes mainly in BRCA1 [7].

Pathogenic mutations in the BRCA1 and BRCA2 genes confer high risks of breast, ovarian, and contralateral BC. In a recent study the average cumulative risks by age 70 years for BRCA1 carriers were estimated to be 60 % (95 % confidence interval [CI] = 44 to 75 %) for BC, 59 % (95 % CI = 43 to 76 %) for OC, and 83 % (95 % CI = 69 to 94 %) for contralateral BC [8]. For BRCA2 carriers, the corresponding risks were 55 % (95 % CI = 41 to 70 %) for BC, 16.5 % (95 % CI = 7.5 to 34 %) for OC, and 62 % (95 % CI = 44 % to 79.5 %) for contralateral BC [8].

The prevalence of BRCA1 and BRCA2 mutations varies between ethnic groups and geographical areas [9]. At present, more than 1600 mutations in BRCA1 and more than 1900 mutations in BRCA2 have been described [10]. The spectrum of disease-associated BRCA1 and BRCA2 alleles includes frameshift, nonsense, and missense mutations altering protein function, splice mutations leading to truncation, as well as large genomic rearrangements [11, 12]. The majority of germline mutations identified in BRCA1 and BRCA2 are “private” or family-specific [12]. However, several examples of founder mutations have been described in certain geographical areas and ethnic communities where the BRCA1 and BRCA2 mutational spectra are limited to a few founders [13]. In fact, founder mutations have been described in Ashkenazi Jews (AJ), Icelandic and Finnish populations, in certain Dutch and French-Canadian communities, and in countries like Turkey, Pakistan, India, etc. [12–14].

Identification of BRCA1/2 mutation carriers allows nondirective clinical decisions to be made in the management of high life time risk of BC/OC including follow-up, prophylactic mastectomy and salpingooophorectomy [7]. Furthermore, mutations in BRCA1/2 have been shown to be predictive of good response to certain treatments, such as cisplatin and Poly (ADP)-Ribose Polymerase (PARP) inhibitors [7].

According to the National Cancer Registry, BC is the most common female malignancy in Bulgaria [15]. Around 3885 women are diagnosed with BC and 1285 die from the disease each year [15]. The first study of 20 Bulgarian familial BC patients was performed in 1998 aiming to develop a screening approach for the BRCA1 gene [16]. No mutations but only benign polymorphisms were identified, most likely due to the small sample size, the low stringency of the selection criteria and the lack of complete analysis of BRCA2. Up to date no other surveys on BRCA1/2 mutations in Bulgarian patients with familial BC/OC have been published.

The lack of sufficient genetic studies for inherited mutations in BRCA1/2 genes in the Bulgarian population impedes the introduction of an effective mutation screening that would identify the individuals at high risk in BC/OC families. The aim of the present work was to
conduct a genetic analysis for BRCA1/2 germline mutations in a cohort of 200 Bulgarian women with BC, fulfilling the recognized international criteria [17, 18]. Direct sequencing of all coding exons and intron-exon junctions of both genes was performed. This is the first comprehensive study in Bulgaria aiming to ascertain the contribution of BRCA1/2 germline mutations to familial BC in the Bulgarian population.

Methods

Participants

In the present study 200 unrelated female patients with primary BC, were selected from the Departments of Surgery and General and Clinical Pathology, University Hospital, “Alexandrovska”, Medical University of Sofia, and the Clinic of Medical Oncology, Specialized Hospital for Active Treatment in Oncology, Sofia, during their treatment and follow up procedures for the period of 2007–2012. All patients were of Bulgarian ethnicity, except one of Jewish origin. In personal interviews all clinical information, histopathology reports and family history were obtained from the patients. Their clinical features are summarized in Table 1. Sixty-one first-degree relatives (8 diagnosed with BC/OC and 53 healthy) of 30 probands possessing a strong family history of BC/OC have been also recruited. They were considered for testing of possibly inherited damaging mutations. The cancer diagnosis of the affected relatives was verified by their personal clinical records.

Probands were selected for BRCA1/2 genetic testing according to their age of diagnosis, family history and tumour characteristics following the recognized Breast Cancer Linkage Consortium (BCLC) and National Comprehensive Cancer Network (NCCN) Criteria, summarized in Additional file 1: Table S1 [17, 18].

The distribution by criteria was as follows: 61 % (n = 122) fulfilled the BCLC criteria, 25 % (n = 50) - the NCCN criteria and 14 % (n = 28) were TNBC. The predominant was the group with early onset (38 %, n = 76), followed by the group with family history of BC (37 %, n = 73) in at least one first or second-degree relative diagnosed under the age of 60 (Table 1). Fifteen % (n = 30) of the patients were diagnosed with BBC, 6 % (n = 12) had a personal history of OC, whereas 5 % (n = 11) – family history of OC in at least one first or second-degree relative diagnosed at any age (Table 1). In addition to the TNBC group (14 %, n = 28) (Table 1), 14 % (24/172) of the patients selected by the BCLC and 2 NCCN criteria were also TNBC.

All deleterious mutations and some of the variants with unknown clinical significance (VUSs) found in the patients were validated in a control group of 96 healthy women matched by age and ethnicity to the patients, without family history of BC/OC. Only VUSs associated with BC in previous studies were screened in the control group.

The control group was collected through the clinical specialists participating in the study and included women undergoing regular prophylactic breast examinations in the University Hospital “Alexandrovska”, Medical University of Sofia, and Specialized Hospital for Active Treatment in Oncology, Sofia. They were defined as healthy based on the results of their last breast examinations signed by breast specialists and in addition were screened for lack of family history of BC/OC.

The study was approved by the Ethics Committee, Medical University of Sofia. All persons confirmed their agreement for participation upon signing an informed consent and donated 10 ml of blood for genetic analysis. The participants have also given consent for publishing their genetic results and relevant clinical data anonymously under a specific ID code assigned.

Genetic analysis

Total genomic DNA was isolated from peripheral blood using a Chemagic Magnetic Separation Module and the Chemagic DNA Blood kits according to the manufacturer’s recommendations (Chemagen, Perkin Elmer).

In order to amplify all coding sequences and exon-intron junctions of the BRCA1/2 genes, we have used a set of 81 primer pairs (33 for BRCA1 and 48 for BRCA2) selected from the BIC database [10] or designed by Exon Primer software [19] (Additional file 2: Table S2). PCR reactions were performed in 1X PCR buffer (Invitrogen), 0.4 mM of each dNTP, 1.5 mM MgCl2, 0.4 pmol of each primer and 1U of Taq DNA polymerase (Invitrogen) with 50 ng of genomic DNA in a final volume of 10 µl. Amplification conditions were identical for all amplicons of both genes, ranging in size from 190 to 720 bp, excluding the specific hybridization temperatures of the primer pairs given in Additional file 2: Table S2 initial denaturation at 94 °C (5 min); followed by 35 cycles of 94 °C (45 s), 51 °C - 63 °C (35 s), 72 °C (30 s) and 72 °C (10 min).

The mutation screening of BRCA1/2 genes was performed using Sanger sequencing. PCR products were purified with ExoSapIT (Affymetrix) according to the manufacturer’s instructions – equal amounts of ExoSap and dH2O (0.4 µl each) were added to 1 µl of PCR product and incubated in a cycler for 30 min at 37 °C, followed by 15 min at 80 °C. Sequencing reactions were carried out with Big Dye® Terminator kit v3.1 (Life Technologies) in a final volume of 10 µl under the following conditions: 96 °C (5 min), 96 °C (20 s), 55 °C (20 s), 60 °C (2 min), 60 °C (5 min).

The fragments were analysed by automated Genetic Analyser ABI 3130xl (Applied Biosystems). Obtained sequences were examined for the presence of mutations by alignment with reference DNA sequence (GenBank U14680 for BRCA1; GenBank U43746 for BRCA2) using
BLAST [20] and SeqScape TM v 2.0 (Applied Biosystems, USA) software packages. Any mutation found was confirmed in a second PCR reaction followed by sequencing in both forward and reverse directions.

In silico analysis of sequence variants
Potential structural and functional effect of the missense VUSs was predicted by the following online tools: PolyPhen 2 (http://genetics.bwh.harvard.edu/pph/), SIFT (http://sift.jcvi.org/) and PROVEAN (http://provean.jcvi.org/index.php) [21–23].

Results
In the current study 200 Bulgarian patients selected by the established genetic testing criteria were screened for mutations in the BRCA1/2 genes by direct sequencing. The mean age of the patients at diagnosis was 49.5 (25–74) years. Thirty eight percent (n = 76) of the patients were under 40, 49.5 % (n = 99) between 41 and 60, and 12.5 % (n = 25) between 61 and 80 years of age (Table 1).

Upon direct sequencing, 13 different damaging mutations were identified, 6 in BRCA1 (four frameshift, one nonsense, one missense) and 7 frameshift in BRCA2 (Table 2). Of those 84.61 % (11/13) were frameshift mutations seen in 18 % (36/200) of the patients. None of the pathogenic mutations was found in the healthy controls.

BRCA1 mutations
In the BRCA1 gene we have identified six unequivocally deleterious mutations of which four frameshift, one nonsense and one missense (Table 2). Among the frameshift mutations two: c.5030_5033delCTAA and c.5263_5264insC with frequencies of 0.5 % and 11 %, respectively, had been previously reported and two, namely c.464delA and c.5397_5403delCCCTTGG with frequencies of 0.5 % each were novel (Table 2). The mutation c.464delA, located in exon 8, was detected in a patient BC134 diagnosed with TNBC at the age of 54 with three cases of BC in her pedigree (Table 3). The second novel mutation c.5397_5403delCCCTTGG in exon 22 was also found in an individual with TNBC (BC205) diagnosed at the age of 51 with family history of BC and PC (Table 3).
The deletion c.5030_5033delCTAA in **BRCA1** exon 17 was observed in one patient (BC194) without family history diagnosed with TNBC at the age of 63 (Table 3). Twenty-two of the patients (11 %) harboured the mutation c.5263_5264insC in **BRCA1** exon 20 (Table 3). Of those six (BC6, BC21, BC99, BC143, BC152 and BC171) had developed both BC and OC, four were diagnosed with BBC (BC7, BC39, BC152 and BC204). Eleven of the cases were TNBC (BC7, BC39, BC152 and BC204). Eleven of the cases were TNBC (BC7, BC39, BC152 and BC204). Eleven of the cases were TNBC (BC7, BC39, BC152 and BC204). Eleven of the cases were TNBC (BC7, BC39, BC152 and BC204).

In addition to the listed above indels we found two deleterious point mutations that had previously been reported (Table 2): one nonsense (c.4603G > T) with a frequency of 0.5 % and one missense (c.181T > G) with a frequency of 1 %. The mutation c.4603G > T was carried by a patient BC37 with early onset (35 years) and in a Jewish patient BC52 with family history of BC, diagnosed with both BBC (at the age of 41/68) and OC (at the age of 59) (Table 4).

The deletion c.7910_7914delCCTTT, located in exon 17, was present in a patient BC19 with early onset, BBC (at the age of 37/41) and family history of BC/OC (Table 4).

The second new **BRCA2** deletion c.9682delA, located in exon 27, was also observed in a patient (BC228) with BBC (Table 4).

### Unclassified variants

Additional 50 sequence variants were identified at **BRCA1** and **BRCA2**, with either benign or unknown pathogenic effect. All of them were named sequence variants due to inconsistency in their classification in the

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**Table 2** **BRCA1/2** damaging mutations in Bulgarian BC patients

| Exon | HGVS nomenclature | BIC nomenclature | Protein nomenclature | Functional domain | Mutation type | Times observed |
|------|-------------------|-----------------|----------------------|-------------------|--------------|---------------|
| 5    | c.181 T > G       | C61G            | p.Cys61Gly           | Ring finger       | Missense     | 2             |
| 8    | c.464delA         | 583delA         | p.Gln155fs           | -                 | Frameshift   | 1             |
| 15   | c.4636G > T       | E1535X          | p.Glu1535Ter         | AD1               | Nonsense     | 1             |
| 17   | c.5030_5033delCTAA| 5149del4        | p.Thr1677_Asn1678delinsHefs | BRCT1/AD2 | Frameshift   | 1             |
| 20   | c.5263_5264insC   | 5382insC        | p.Ser1755delinsSerProfs | linker           | Frameshift   | 22            |
| 22   | c.5397_5403delCCCTTG    | 5515del7  | p.Thr1799delins      | BRCT2/AD2        | Frameshift   | 1             |
| 11   | c.5718_5719delICT  | 5946delICT      | p.Asn1906_Ser1907 = fs | -                | Frameshift   | 1             |
| 11   | c.5851_5854delAGTT | 6079del4       | p.Ser1951_Leu1952delinsTrpfs | -                | Frameshift   | 2             |
| 11   | c.5946delT        | 6174delT        | p.Ser1982Argfs       | BRC repeat?      | Frameshift   | 1             |
| 17   | c.7910_7914delCCTTT| 8138del5       | p.Ala2637_Phe2638delinsAlafs | Helical Domain | Frameshift   | 1             |
| 20   | c.8532_8533delAA  | 8760delAA       | p.Glu2844fs          | Tower             | Frameshift   | 1             |
| 23   | c.9098_9099insA   | 9326insA        | p.Thr3033delinsThrSerfs | OB2               | Frameshift   | 4             |
| 27   | c.9682delA        | 9908delA        | p.Gln3227fs          | -                 | Frameshift   | 1             |

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| BCN | HGVS nomenclature | Diagnosis and age of onset | ER | PR | HER2 | TNM | Grade | Stage (age of onset) | Other cancer in patient | Family history of cancer and age of diagnosis |
|-----|-----------------|--------------------------|----|----|-----|-----|------|---------------------|------------------------|-------------------------|
| 3   | c.5263_5264insC | BC (36)                  | NA | NA | NA  | NA  | NA   | NA                  | DC (41)                | Grandmother - BC (NA)  |
| 6   | c.5263_5264insC | IDC (43)                 | NA | NA | NA  | pT2N0Mx | NA | NA                      | OC (45)                | Mother – BC (50), sister – BBC (42/53), grandmother – BC and OC (50) |
| 7   | c.5263_5264insC | BBC (42)                 | 3+/− | 3+/− | 1+/1+ | pT1N0M0/pT1bpN1M0 | G2/G2 | I/II                      |                        | Grandmother - BC (76), aunt BC(81), father seminoma (NA), cousin with cancer of the oral cavity (NA) |
| 17  | c.5263_5264insC | IDC (34)                 | 2+ | 2+ | NA  | NA  | G2   | NA                  |                        | No                      |
| 21  | c.5263_5264insC | IDC (48)                 | NA | NA | NA  | NA  | NA   | NA                  | OC (48)                | Grandfather - GC (70), grandfather – BT (NA), PC (NA), aunt - CGO (50), cousin - BC (54) |
| 28  | c.181 T > G     | BBC (52/60)              | NA | NA | NA  | NA  | G2   | NA                  | OC (52)                | Brother with bone cancer (47) |
| 37  | c.4603G > T     | IDC (37)                 | - | - | -   | pT1cN1M0 | G2 | II                      | FtC (41)               | Grandfather - PC (76), grandfather - LC (69), grandmother - CRC (60) |
| 39  | c.5263_5264insC | BBC (37/55)              | NA/- | NA/- | NA/- | NA/- | NA   | NA                  |                        | Uncle - PrC (66)       |
| 73  | c.5263_5264insC | IDC (34)                 | NA | NA | NA  | pT2pN0M0 | G3 | II                      |                        | Mother - GC (70), mother – BBC (27/30), uncle – leukemia (67), grandmother - Clv (75) |
| 79  | c.5263_5264insC | IDC (42)                 | NA | NA | 1+  | NA  | NA   | NA                  |                        | Grandmother – unknown cancer (>80), mother-CrC (NA), mother’s sister – Paget’s disease of the nipple (NA) |
| 99  | c.5263_5264insC | IDC (40)                 | NA | NA | NA  | pT1N0M0 | G2 | NA                      | OC (46)                |                        |
| 111 | c.5263_5264insC | ACC (36)                 | - | - | -   | NA  | G1   | NA                  |                        | No                      |
| 121 | c.5263_5264insC | IDC (34)                 | - | - | -   | pT1N0M0 | G3 | I                       |                        | Father – pelvic cancer (NA), cousin - BC (30) |
| 132 | c.181 T > G     | IDC (39)                 | - | - | -   | pT1pN0M0 | G2 | I                       |                        | Grandmother - BC (60), mother - BBC (32/40) |
| 134 | c.464delA       | IDC (54)                 | - | - | -   | pT1N1M0 | G3 | II                      |                        | Aunt - BC (47), grandmother and grandmother’s sister – BC/NA, father - LC (NA) |
| 140 | c.5263_5264insC | IDC (32)                 | 1+ | 2+ | 2+  | pT2pN0M0 | G2 | IIa                     |                        | Mother - BBC (31/37), grandmother - EC, CRC (50/53), grandfather - LC (59), grandmother - BC (NA) |
| 142 | c.5263_5264insC | IDC (31)                 | NA | NA | NA  | NA  | G2   | NA                  |                        | No                      |
| 143 | c.5263_5264insC | IDC (51)                 | NA | NA | NA  | pT2N0M0 | G3 | I                       | OC (51)                | Uncle - PC (68), grandmother LC (44), cousin - OC (37) |
| 152 | c.5263_5264insC | BBC (41)                 | NA | NA | NA  | NA  | NA   | NA                  | OC (58)                | Mother - BBC (NA)      |
| 155 | c.5263_5264insC | BC (29)                  | NA | NA | NA  | NA  | NA   | NA                  |                        | Grandmother - BC, ThC (50/70), cousin - BC (50), cousin - OC (48) |
| 161 | c.5263_5264insC | IDC (43)                 | NA | NA | NA  | NA  | G2   | NA                  |                        | Mother – OC (62)       |
| 164 | c.5263_5264insC | IDC (45)                 | NA | NA | NA  | NA  | NA   | NA                  |                        | Mother - BC (NA)       |
| 171 | c.5263_5264insC | BC (53)                  | NA | NA | NA  | NA  | NA   | NA                  | OC (NA)                | Grandmother - BC (43), mother - BC (53) and OC (63) |
| 175 | c.5263_5264insC | MBC (33)                 | NA | NA | NA  | pT1cN2M0 | G2 | II                      |                        | No                      |
| 194 | c.5030_5033     | IDC (63)                 | NA | NA | NA  | pT1N0M0 | G2 | NA                      |                        | No                      |
| Case | Mutation | Clinical Data | Histology | Grade | Family History |
|------|----------|---------------|-----------|-------|----------------|
| 190  | c.5263_5264insC IDC (33) | - - - | pT1bN0M0 | G2 | I Grandmother - BC (47), mother - OC (52), grandfather - CRC (62) |
| 204  | c.5263_5264insC BBC (35/48) | NA NA NA | NA NA | Mother with bladder cancer in doubt |
| 205  | c.5397_5403 IDC (51) | - - - | pT1pN1M0 | G3 | II Grandmother - BC (30), father - PC (NA) |

**Table 3:** Clinical data of the carriers of damaging *BRCA1* mutation. (Continued)

- **Case:** Case identifier.
- **Mutation:** Type and location of the mutation.
- **Clinical Data:** Details about the case.
- **Histology:** Histological type of the cancer.
- **Grade:** Grade of the cancer.
- **Family History:** Details about family members with cancer.

**Abbreviations:**
- BC: Breast cancer
- BBC: Bilateral breast cancer
- IDC: Invasive ductal carcinoma
- ILC: Invasive lobular carcinoma
- IDLC: Infiltrating ductal/lobular cancer
- MBC: Medullary breast cancer
- ACC: Adenoid cystic carcinoma
- ThC: Throat cancer
- LyC: Laryngeal cancer
- PC: Prostate cancer
- LC: Lung cancer
- CRC: Colorectal carcinoma
- PrC: Pancreatic cancer
- CLv: Liver cancer
- GC: Gastric cancer
- OC: Ovarian cancer
- BT: Brain tumor
- EC: Endometrial cancer
- CGO: Cancer of genital organs
- DC: Dysplasia of the cervix
- FtC: Fallopian tube cancer
- NA: Not available

*Note:* **delCCCTTGG**

**Reference:** Dodova et al. BMC Cancer (2015) 15:523
| BCNP | HGVSnomenclature | Diagnosis and age of onset | ER   | PR   | HER2 | TNM | Grade | Stage | Other cancer in patient | Family history of cancer and age of diagnosis |
|------|------------------|---------------------------|------|------|------|-----|-------|-------|-------------------------|-----------------------------------------------|
| 19   | c.7910_7914      | BBC (31/37)               | NA/NA| NA/NA| NA/NA| NA/NA| NA/NA | NA/NA| Sister - BBC (41/49), father - GC (NA), aunt - OC (NA) |
| 24   | c.9682delA       | BBC (52)                  | NA   | NA   | NA   | NA   | NA   | NA   | NA                      | NA                                            |
| 32   | c.9098_9099insA  | IDC (50)                  | -    | -    | 3+   | NA   | G3   | NA   | Aunty - BC (NA)          | Mother - BC (36), sister - BC (46) and CRC (65), father - LC, ethnicity - Jewish |
| 52   | c.5946delT       | BBC (41/68)               | NA/NA| NA/NA| NA/NA| NA/NA| NA/NA| NA/NA| OC (59)                 | Mother - BC (36), daughter - BC (65) and CRC (65), father - LC, ethnicity - Jewish |
| 58   | c.5851_5854      | IDC (48)                  | 3+   | 3+   | 2+   | pT1N0Mx| G3   | NA   | Mother - BC (NA), brother and sister of the mother - CRC (NA), father - LC (NA), cousin - CRC (NA), a brother of the father - CLv (NA) and his children - BC (59) and - LC (50) |
| 76   | c.5851_5854      | IDLC (53)                 | -    | -    | -    | pT1pN0M0 | G2   | NA   | Cousin - BC (65), father - StC (54) |
| 81   | c.9098_9099insA  | BC (59)                   | NA   | NA   | NA   | NA   | NA   | NA   | Sister - BC (52), aunt - BC (67) |
| 85   | c.9098_9099insA  | IDC (52)                  | 3+   | -    | -    | pT1pN2pMx| G3   | III  | Aunty - BC (42), grandfather - head and neck cancer (NA) |
| 87   | c.8532_8533      | BBC (30/37)               | 3+   | -    | NA   | pT1bPnM0/ | G2/2 | I/I  | Mother BBC (NA)          |
| 88   | c.9098_9099insA  | IDLC (61)                 | 3+   | /3+  | /1+  | pT1bPnM0 | NA   |     | Grandfather - ThC (48), mother - BC (60), two cousins - BC (44/47) |
| 90   | c.5718_5719      | IDC (35)                  | 2+   | 3+   | 3+   | pT2pN2M0 | G2   | III  | Grandfather - LyC (NA), grandmother - unclear malignant disease, most likely melanoma (NA) |

**BCNP** - case identifier; **BC** – breast cancer, **BBC** – bilateral breast cancer, **IDC** – invasive ductal carcinoma, **ILC** – invasive lobular carcinoma, **ILDC** – infiltrative ductal lobular cancer, **MBC** – medullary breast cancer, **ACC** – adenoid cystic carcinoma, **ThC** – throat cancer, **LyC** – laryngeal cancer, **PC** – prostate cancer, **LC** – lung cancer, **CRC** – colorectal carcinoma, **PCc** – pancreatic cancer, **CLv** – cancer of the liver, **GC** – gastric cancer, **OC** – ovarian cancer, **BT** – brain tumor, **EC** – endometrial cancer, **CGO** – cancer of genital organs, **DC** – dysplasia of the cervix, **FtC** – fallopian tube cancer, **NA** – not available, “-“-negative for ER, PR and HER2
### Table 5 Unclassified BRCA1/2 variants in Bulgarian BC patients

| Exon | HGVS nomenclature | BIC nomenclature | Protein nomenclature | Functional domain | Mutation type | MAF in Bulgarian patients | Analysed patients (n) | MAF in healthy controls | Analysed healthy controls (n) | MAF in Europeans |
|------|-------------------|------------------|-----------------------|-------------------|--------------|--------------------------|----------------------|--------------------------|-------------------------------|------------------|
| **BRCA1** | | | | | | | | | | |
| 5 | c.139 T > G | C47G | p.Cys47Gly | ring finger | missense | 0.002 | 200 | 0.000 | 96 | NA |
| 8 | c.536A > G | Y179C | p.Tyr179Cys | - | missense | 0.002 | 194 | 0.000 | 96 | 0.001 |
| 11 | c.736 T > G | L246V | p.Leu246Val | - | missense | 0.002 | 200 | - | - | 0.002 |
| 11 | c.1067A > G | Q356R | p.Gln356Arg | - | missense | 0.072 | 194 | 0.052 | 96 | 0.049 |
| 11 | c.1456 T > C | F486L | p.Phe486Leu | - | missense | 0.002 | 200 | - | - | 0.001 |
| 11 | c.1648A > C | N550H | p.Asn550His | - | missense | 0.002 | 200 | 0.000 | 96 | 0.001 |
| 11 | c.2077G > A | D693H | p.Asp693Asn | - | missense | 0.065 | 192 | 0.083 | 96 | 0.097 |
| 11 | c.2612T > C | P871L | p.Pro871Leu | - | missense | 0.350 | 193 | 0.391 | 96 | 0.336 |
| 11 | c.3113A > G | E1038G | p.Glu1038Gly | - | missense | 0.335 | 195 | 0.349 | 96 | 0.358 |
| 11 | c.3119G > A | S1040N | p.Asn1040Ser | - | missense | 0.023 | 195 | 0.026 | 96 | 0.058 |
| 11 | c.3548A > G | K1183R | p.Lys1183Arg | - | missense | 0.339 | 193 | 0.302 | 96 | 0.358 |
| 11 | c.3999 T > C | V1333A | p.Val1333Ala | AD1 | missense | 0.002 | 200 | 0.000 | 96 | - |
| 16 | c.4837A > G | S1613C | p.Ser1613Cys | AD2 | missense | 0.323 | 193 | 0.318 | 96 | 0.314 |
| 16 | c.4956G > A | M1652I | p.Met1652Ile | BRCT1/AD2 | missense | 0.036 | 193 | 0.016 | 96 | 0.005 |
| 9 | c.591C > T | C197C | p.Cys197Cys | - | cds-synon | 0.007 | 193 | 0.000 | 96 | 0.001 |
| 11 | c.2082C > T | S694S | p.Ser694Ser | - | cds-synon | 0.323 | 192 | 0.318 | 96 | 0.280 |
| 11 | c.2311 T > C | L771L | p.Leu771Leu | - | cds-synon | 0.233 | 191 | - | - | 0.332 |
| 13 | c.4308 T > C | S1436S | p.Ser1436Ser | AD1 | cds-synon | 0.337 | 194 | - | - | 0.332 |
| 7 | c.441 + 36_441 + 38delCTT | IVS5 + 36delCTT | - | - | IVS | 0.194 | 195 | 0.354 | 96 | NA |
| 8 | c.442-34C > T | IVS5-34 C > T | - | - | IVS | 0.230 | 194 | 0.224 | 96 | 0.257 |
| 9 | c.548-58delCTT | S548delCTT | - | - | IVS | 0.196 | 193 | 0.385 | 96 | 0.331 |
| 15 | c.4485-63C > G | IVS14-63C > G | - | - | IVS | 0.330 | 197 | - | - | 0.321 |
| 18 | c.5075-53C > T | IVS17-53 C > T | - | - | IVS | 0.010 | 194 | - | - | 0.034 |
| 20 | c.5277 + 56delGTA | IVS20 + 56insGTA | - | - | IVS | 0.002 | 200 | 0.000 | 96 | - |
| **BRCA2** | | | | | | | | | | |
| 10 | c.865A > C | N289H | p.Asn289His | - | missense | 0.050 | 185 | - | - | 0.031 |
| 10 | c.978C > A | S326R | p.Ser326Arg | - | missense | 0.010 | 185 | - | - | 0.001 |
| 10 | c.1114A > C | H372N | p.Asn372His | - | missense | 0.283 | 185 | - | - | 0.300 |
| 11 | c.2971A > G | N991D | p.Asn991Asp | - | missense | 0.050 | 185 | - | - | 0.031 |
| 11 | c.3515C > T | S1172L | p.Ser1172Leu | - | missense | 0.002 | 185 | 0.000 | 96 | 0.001 |
| 11 | c.5744C > T | T1915M | p.Thr1915Met | - | missense | 0.017 | 200 | 0.016 | 96 | 0.032 |
| 11 | c.6100C > T | R2034C | p.Arg2034Cys | - | missense | 0.002 | 200 | - | - | 0.018 |
| 11 | c.6322C > T | R2108C | p.Arg2108Cys | - | missense | 0.002 | 200 | - | - | 0.001 |
| 15 | c.7469 T > C | I2490T | p.Ile2490Thr | Helical Domain | missense | 0.002 | 185 | - | - | 0.000 |
| 23 | c.9104A > C | Y3035S | p.Tyr3035Cys | OB2 | missense | 0.005 | 200 | 0.005 | 96 | NA |
| 11 | c.4146_4148delAGA | E1382del | delinsAsp | - | Cds_synon | 0.002 | 200 | - | - | NA |
| 10 | c.1365A > G | S455S | p.Ser455Ser | - | Cds_synon | 0.030 | 185 | - | - | 0.031 |
| 11 | c.2229T > C | H743H | p.His743Asn | - | Cds_synon | 0.035 | 184 | - | - | 0.031 |
| 11 | c.3396A > C | K1132K | p.Lys1132Asn | - | Cds_synon | 0.285 | 182 | - | - | 0.250 |
| 11 | c.3807 T > C | V1269V | p.Val1269Val | - | Cds_synon | 0.189 | 185 | - | - | 0.226 |
The spectrum of unclassified variants included missense, synonymous and intronic variants, as well as one inframe deletion (Table 5).

In total 24 missense VUSs were identified, of which 14 in BRCA1 and 10 in the BRCA2 (Table 5). According to their minor allele frequencies (MAFs) in the patients they can be divided into two groups: rare variants with MAFs between 0.0025 and 0.005, and frequent variants with MAFs > 0.005. We have compared the MAFs of the unclassified missense variants found among patients with other Europeans [24] and with healthy controls (only for the variants associated with BC in previous studies). The studied variants in the control group included VUSs in exons 5 (c.139 T > G); 7 (c.441 + 36_441 + 38delCTT); 8 (c.536A > G, c.442-34C > T); 9 (c.591C > T, c.548-58delT); 11 (c.1067A > G, c.1648A > C, c.2077G > A, c.2612C > T, c.3113A > G, c.3548A > G, c.3999 T > C, c.2082C > T); 16 (c.4837A > G, c.4956G > A); and 20 (c.5277 + 56delCCTT) of BRCA1 and the variants in exons 11 (c.3515C > T, c.5744C > T); 17 (c.7806-14 T > C); and 23 (c.9104A > C) of BRCA2 (Table 5). Variants for which no information for association with BC was found in the databases were not screened in the healthy controls but were included in Table 5. In addition in silico analysis was conducted in order to predict potential deleterious effect of all detected missense VUSs (Table 6).

In order to rule out the possibility of these missense variants being benign variants seen in healthy controls, we analyzed the MAFs of the unclassified missense VUSs found among patients with other Europeans (only for the variants associated with BC in previous studies). The studied variants in the control group included VUSs in exons 5 (c.139 T > G); 7 (c.441 + 36_441 + 38delCTT); 8 (c.536A > G, c.442-34C > T); 9 (c.591C > T, c.548-58delT); 11 (c.1067A > G, c.1648A > C, c.2077G > A, c.2612C > T, c.3113A > G, c.3548A > G, c.3999 T > C, c.2082C > T); 16 (c.4837A > G, c.4956G > A); and 20 (c.5277 + 56delCCTT) of BRCA1 and the variants in exons 11 (c.3515C > T, c.5744C > T); 17 (c.7806-14 T > C); and 23 (c.9104A > C) of BRCA2 (Table 5). Variants for which no information for association with BC was found in the databases were not screened in the healthy controls but were included in Table 5. In addition in silico analysis was conducted in order to predict potential deleterious effect of all detected missense VUSs (Table 6).

Six rare BRCA1 missense variants were seen in patients (Table 5): c.139 T > G, c.536A > G, c.736 T > G, c.1456 T > C, c.1648A > C and c.3999 T > C. Of those c.3999 T > C, causing a replacement of alanine to valine at codon1333 has never been reported. Even though c.3999 T > C was observed in a patient BC20 with BC and early onset in the absence of other pathogenic mutations, the in silico analysis did not confirm its pathogenic effect (Table 6).

The missense variants c.139T > G had been predicted to be deleterious in previous functional studies [25]. In our study it was found in one patient (0.5 %) with TNBC BC10 and appeared to be clinically important according to wo prediction programs (POLYPHEN 2 and SIFT), while PROVEAN predicted it as neutral (Table 6).

One patient with family history of BC117 was the only carrier of three rare missense variants c.536A > G, c.1456T > C and c.1648A > C, which were not seen in the control group (Table 5). Another rare missense BRCA1 variant c.736T > G, listed as variant of unknown significance (VUS) in BIC [10] and neutral in UMD (Universal Mutation Database) [26], was found in patient BC30 with BBC (Table 5).

Eight missense variants were observed in BRCA1 with MAF>0.005 in both patients and controls (Table 5). They were designated as VUS in BIC and neutral in UMD databases (Table 5). Only two of them (c.1067A > G and c.3113A > G) appeared to be deleterious according to the conducted in silico analysis (Table 6).

Four synonymous and 6 intronic variants were also detected in the BRCA1 gene (Table 2). Of those c.5277 + 62_c.5277 + 64delGTTA and c.5277 + 64delGTTA had not previously been reported and was seen in two patients (1 %) but not in the control group (Table 5). The variant c.591C > T was more frequent in patients (MAF = 0.007), but was not found in the control group (Table 5). and was seen in two patients (1 %) but not in the control group (Table 5).

Table 5 Unclassified BRCA1/2 variants in Bulgarian BC patients (Continued)

| #  | VUS in BRCA1 or BRCA2 | MAF in healthy controls | MAF in patients | VUS in BRCA1 or BRCA2 | MAF in healthy controls | MAF in patients |
|----|----------------------|-------------------------|-----------------|----------------------|-------------------------|-----------------|
| 14 | c.7242A > G          | 0.184                   | 184             | c.7242A > G          | 0.190                   |                 |
| 2  | c.-26G > A           | 0.247                   | 190             | c.-26G > A           | 0.199                   |                 |
| 27 | c.*105A > C          | 0.183                   | 185             | c.*105A > C          | 0.274                   |                 |
| 4  | c.425 + 67A > C      | 0.050                   | 184             | c.425 + 67A > C      | 0.031                   |                 |
| 4  | c.425 + 147G > T     | 0.040                   | 184             | c.425 + 147G > T     | 0.062                   |                 |
| 5  | c.426-89 T > C       | 0.040                   | 184             | c.426-89 T > C       | 0.031                   |                 |
| 8  | c.681 + 56C > T      | 0.060                   | 185             | c.681 + 56C > T      | 0.133                   |                 |
| 11 | c.6841 + 80_6841 + 83| 0.289                   | 185             | c.6841 + 80_6841 + 83| 0.294                   |                 |
|    | delTTAA              | 0.032                   | 186             | delTTAA              | 0.043                   |                 |
| 13 | c.7007 + 134_7007 + 135insTTATAAAAT| - | - | c.7007 + 134_7007 + 135insTTATAAAAT | - | - |
| 15 | c.7617 + 28C > A     | 0.002                   | 200             | c.7617 + 28C > A     | 0.002                   |                 |
| 17 | c.7806-14 T > C      | 0.320                   | 187             | c.7806-14 T > C      | 0.469                   | 96              | 0.465 |
Five rare missense variants (MAF < 0.005) were identified in BRCA2 (Table 5). Three of them c.6322C > T, c.7469 T > C and c.9104A > C were classified as VUS in both BIC [10] and UMD [26] databases, while c.3515C > T and c.6101G > A were designated as VUS only in BIC (Table 5). In UMD c.3515C > T was suggested to be likely neutral but no information was available for c.6101G > A (Table 5). The in silico analysis predicted pathogenic effect of both c.3515C > T and c.6101G > A (Table 6).

We found four frequent (MAF > 0.005) missense variants in BRCA2 (Table 5). None of them was predicted to be pathogenic upon in silico analysis (Table 6). In addition we detected one in frame deletion of glutamate in exon 11- c.4146_4148delAGA (MAF = 0.0025), which was classified as VUS in both BIC [10] and LOVD (Leiden Open Variation Database) [27].

The other BRCA2 sequence variants were observed with high frequencies in patients (MAF > 0.005), similar to those found in other Europeans (Table 5). Among them seven were synonymous replacements; two variants – c.2612C > T and c.3113A > G were localized in 5′-UTR- and 3′-UTR, respectively and 8 were intronic. One of the intronic variants c.7617 + 28C > A was novel (Table 5).

**Discussion**

This is the first comprehensive study aiming to ascertain the contribution of BRCA1/2 germline mutations to BC development in the Bulgarian population, where 1285 women die from the disease each year [15]. The thorough mutation screening of all coding sequences and exon-intron junctions of BRCA1/2 genes in a cohort of 200 Bulgarian women with primary invasive BC and with personal or family history of BC/OC, selected according to the recognised international criteria [17, 18] led to the identification of pathogenic mutations in 19.5 % of them (39/200).

We have found 13 unequivocally deleterious mutations of which 6 in BRCA1 and 7 in BRCA2 gene (Table 2). The predominant mutations seen in 18 % of the studied group of patients were frameshift mutations leading to truncated proteins with impaired function. In total.

| Exon | HGVS nomenclature | BRCA1 nomenclature | BIC | UMD | POLYPHEN2 | SIFT | PROVEAN |
|------|-------------------|---------------------|-----|-----|-----------|------|---------|
| 5    | c.139 T > G       | C47G                | unknown | - | probably damaging | damaging | neutral |
| 8    | c.536A > G        | Y179C               | unknown | neutral | damaging | tolerated | neutral |
| 11   | c.736 T > G       | L246V               | unknown | neutral | probably damaging | tolerated | neutral |
| 11   | c.1067A > G       | Q356R               | unknown | neutral | probably damaging | damaging | deleterious |
| 11   | c.1456 T > C      | F486L               | unknown | neutral | benign | tolerated | neutral |
| 11   | c.1648A > C       | N550H               | unknown | neutral | probably damaging | damaging | deleterious |
| 11   | c.2077G > A       | D693H               | no | neutral | benign | tolerated | deleterious |
| 11   | c.2612C > T       | P871L               | no | neutral | benign | tolerated | neutral |
| 11   | c.3113A > G       | E1038G              | no | neutral | possibly damaging | damaging | neutral |
| 11   | c.3119G > A       | S1040N              | unknown | neutral | probably damaging | damaging | neutral |
| 11   | c.3548A > G       | K1183R              | no | neutral | benign | tolerated | neutral |
| 11   | c.3999 T > C      | V1333A              | - | - | possibly damaging | tolerated | neutral |
| 16   | c.4837A > G       | S1613C              | no | neutral | benign | damaging | neutral |
| 16   | c.4956G > A       | M1652I              | unknown | neutral | benign | tolerated | neutral |

**Table 6** Assessment of the clinical effect of unclassified BRCA1/2 missense variants detected in Bulgarian BC patients

Five rare missense variants (MAF < 0.005) were identified in BRCA2 (Table 5). Three of them c.6322C > T, c.7469 T > C and c.9104A > C were classified as VUS in both BIC [10] and UMD [26] databases, while c.3515C > T and c.6101G > A were designated as VUS only in BIC (Table 5). In UMD c.3515C > T was suggested to be likely neutral but no information was available for c.6101G > A (Table 5). The in silico analysis predicted pathogenic effect of both c.3515C > T and c.6101G > A (Table 6).
11 (84.6 %, 11/13) indels were seen, 4 in BRCA1 and 7 in BRCA2. The other two mutations identified were nonsense and missense mutation in BRCA1, each accounting for 7.7 % (1/13) of all disease causing mutations.

The median age of diagnosis was 46 (range 29–63 years) in the 28 BC with a BRCA1 mutation (Table 3) and 45.5 (range 30–61 years) in the 11 BC with a BRCA2 mutation (Table 4), compared with a median age of diagnosis of 49.6 years (range 25–74 years) for the 161 cases without a mutation. A BRCA1 mutation was found in 18.4 % (14 of 76) women diagnosed with BC at or under the age of 40 compared to 11.2 % (14 of 124) of women diagnosed at a later age (Table 4); a BRCA2 mutation was found in 4 % (3 of 76) women diagnosed with BC at or under the age of 40 compared to 6.5 % (8 of 124) of women diagnosed at a later age (Table 4). A mutation was present in 26.8 % (22 of 82) BC patients with a positive family history and in 14.4 % (17 of 118) of women with a negative family history.

**BRCA1 mutations**

The most prevalent mutation observed in 22 patients (11 %) was BRCA1 c.5263_5264insC, a known Slavic mutation with founder effect in Eastern European as well as AJ communities, (Table 2). This frameshift mutation is the second most frequently reported in BIC database [10]. It has been found with various frequencies in high risk BC/OC families from Poland (34 %), Russia (14 %), Hungary (14 %), Slovenia (13 %), AJ (10 %), Greece (8 %), Germany (4 %), and Italy (3 %) [13]. The c.5263_5264insC was not found in Spain and Portugal and it has been observed with a low frequency in the Netherlands, Belgium and Scandinavian countries [13]. In Russia, Belarus, Poland, Latvia, Czech Republic, Greece and Lithuania it accounts for respectively 94 %, 73 %, 60 %, 55 %, 37–52 %, 46 % and 34 % of all BRCA1 mutations [13].

Being a founder mutation in Greece [28], c.5263_5264insC has been detected in other Balkan countries such as Romania, Turkey, Serbia, Croatia, and Macedonia but without a founder effect [29–33]. Apparently Bulgaria is the second Balkan country in which the most prevalent Slavic mutation c.5263_5264insC has a founder effect accounting for a very high proportion, about 76 % (22/29) from all detected BRCA1 mutations (Table 3), with high intermediate frequency (11 %) compared to the other Central and Eastern European countries [13].

Originally c.5263_5264insC was described as a founder mutation in AJ, but recent haplotype analysis suggested that it most likely originated in Northern Russia or possibly Scandinavia, between 1800 and 1500 years ago, and was subsequently spread to the various populations from East to West and nearly worldwide [34]. A common ancestor for families with c.5263_5264insC mutation, reported from Europe, Brazil, North America and India is evident [34–36].

We suppose that in Bulgaria the mutation c.5263_5264insC also has a Slavic origin since the modern Bulgarian nation originated as a mixture of Slavic, Thracian and Bulgar tribes more than 1300 years ago. A detailed haplotype analysis is necessary to prove this hypothesis.

The increased risk of BC and OC in c.5263_5264insC mutation carriers has been estimated at 67 % (95 % CI, 36–83 %) and 33 % (95 % CI, 8–50 %), respectively [5]. At a molecular level it leads to a stop codon at position 1829 and respectively to a truncated protein that lacks its C-terminal BRCT motif. In our study the presence of the c.5263_5264insC mutation in the patients was associated with early onset in 9 patients (BC3, BC39, BC140, BC111, BC121, BC142, BC155, BC190 and BC204), BBC in 4 patients (BC7, BC39, BC152 and BC204), two subsequent events of BC and OC in 6 patients (BC6, BC21, BC99, BC143, BC152 and BC171) and/or TNBC in 11 patients (BC73, BC111, BC121, BC142, BC143, BC155, BC161, BC164, BC171, BC175 and BC190) (Table 3). Several carriers were also identified among the relatives of the patients with c.5263_5264insC (one individual with OC, another with both BC and OC, as well as three healthy individuals).

The mutation c.5030_5033delCTAA located in BRCA1 exon 17 is a prevalent frameshift mutation reported in at least five French families with BC/OC and most likely originating from a common ancestor [13]. It has been also detected in BC patients with early onset from USA (0.35 %, 1/282) and Taiwan (2.8 %, 1/36) [37, 38]. We observed the mutation in one patient (0.5 %, 1/200) with TNBC (BC194) diagnosed at the age of 63 (Table 4).

According to BIC database [10] the majority of the pathogenic mutations in BRCA1/2 are frameshift mutations (around 70 %), while nonsense and missense mutations contribute with around 10 % each. In the present study we have identified only two previously reported BRCA1 point mutations with clear pathogenic effects: c.181T > G and c.4603G > T, located in exon 5 and 15, respectively (Table 2). The mutation c.4603G > T was reported several times in BIC as clinically important and it was found in Non-AJ patients from America and Venezuela [10, 39]. Among the studied Bulgarian patients c.4603G > T was observed only once (0.5 %) and was associated with early onset BC (BC37, Table 3).

Following the most prevalent mutation c.5263_5264insC, the missense variant c.181T > G has been recognised as the second most frequent mutation in Poland and other European countries among BC/OC families [13]. These two founder mutations accounted for 70–90 % of the BRCA1 mutations found in the Polish population, 80 % in Hungary and 28 % in Germany, respectively [13]. Located in the RING domain of BRCA1, c.181T > G has been found to
impede the coordination of the zinc ions upon binding to the protein in functional studies [25].

In Austria, Slovenia, and Czech Republic the families with c.181T > G mutation represented 15 %, 18 % and 9 %, respectively, of all families carrying BRCA1 mutations [13]. It has been also known as one of the three founder mutations in Byelorussian population with frequencies of 1 % in unselected BC families and 0.2 % in control individuals [40]. The mutation was detected at a very low rate in our neighboring countries such as Greece, Serbia and Romania, as well as in Croatia, Austria, Slovakia, Ukaine, Latvia, Lithuania and Russia [13]. In our study c.181T > G was found as a disease causing mutation in two patients (1 %), with early onset (BC132) and BBC (BC28), respectively (Table 3).

We have identified two new frameshift mutations in the BRCA1 gene: c.464delA and c.5397_5403delCCTTTGG with 0.5 % frequency each (Table 2). The mutation c.464delA, located in exons 8, leads to a truncated protein, lacking its Coiled Coil and BRCT domains and was detected in a patient BC134 with TNBC diagnosed at the age of 54 with three cases of BC in her pedigree (Table 3). Three of her healthy first-degree relatives (sister, and 2 sons) were also carriers of the mutation (Additional file 3: Figure S1). The second novel mutation c.5397_5403delCCTTTGG in exon 22 residing in the BRCT motif leads to a truncated protein without C-terminal BRCA2/AD2 domain. It was found in an individual BC205 with TNBC diagnosed at the age of 51 with family history of BC (Table 3, Additional file 4: Figure S2).

**BRCA2 mutations**

The second in frequency (2 %) mutation observed in our study was the insertion c.9098_9099insA, located in exon 23 of BRCA2 gene. It has been reported as one of the most frequent BRCA1/2 mutations in Germany with a possible founder effect [13]. Together with c.5946delT it also accounted for 50 % of all BRCA2 mutations in BC/OC families from Hungary [13]. The consequence of this mutation is a premature stop codon at position 3042 that leads to a truncated protein losing its C terminal OB3 and NLC motifs and thus the ability to bind a DSS1 protein that regulates its repairing function (Table 2). All four carriers of the insertion c.9098_9099insA in our study (BC32, BC81, BC85 and BC88) had family history of BC, and were diagnosed with BC between 50 and 61 years of age. One of them (BC88) developed TNBC (Table 4).

Three of the observed known frameshift mutations were located in exon 11 of BRCA2 (Table 2): c.5851_5854delAGTT, c.5946delT, and c.5718_5719delCT. The deletion c.5851_5854delAGTT has been detected with a low frequency in cohorts of Italian, American Asian and Indian BC/OC patients [41, 42]. In the present investigation we have identified two (1 %) unrelated Bulgarian patients harbouring c.5851_5854delAGTT deletion. One of the patients (BC76) with a family history of BC and stomach cancer was diagnosed with TNBC at the age of 53, while the other (BC58) developed BC at the age of 48 and had a family history of BC and CRC (Table 4).

The c.5946delT was found in one patient with Jewish origin and family history of BC (BC52), who had developed both BBC at the age of 41/68 and OC at the age of 59 (Table 4). A healthy daughter of the patient also harboured the mutation. The c.5946delT deletion has been recognised as one of the three founder mutations in AJ, together with the two BRCA1 indels c.68_69delAG and c.5263_5264insC [13]. The mutation c.5946delT has been also detected in non-Jewish populations such as the Hungarian BC/OC families [13].

The third mutation, observed in exon 11 of BRCA2 c.5718_5719delCT, was reported several times in BIC database [10]. It has been found with a very low frequency in patients with BC, OC and PC from Germany, UK and North America [10, 43]. The mutation was found with similar low frequency (0.5 %) in our study and correlated with early onset (BC90, Table 4).

Another recurrent mutation according to BIC database [10], c.7910_7914delCCTTT, located in BRCA2 exon 17, was identified in a proband BC19 (0.5 %) with early onset BBC (at the age of 37/41) with family history of BC/OC (Table 4). A cousin of the patient also harboured the mutation and possessed identical clinical phenotype. Interestingly, c.7910_7914delCCTTT has been associated with BBC in other European populations as well [40]. It was first reported in UK and was further observed with low frequencies in patients with BBC from Germany and Denmark and with a frequency of 3.6 % (1/27) among males with sporadic BC in the Polish population [44, 45].

Two novel frameshift mutations were found in BRCA2: c.8532_8533delAA and c.9682delA with frequency of 0.5 % each (Table 2). The mutation c.8532_8533delAA, located in exon 20, was found in a patient BC87 with BBC (at the age of 30 and 37) and early onset (Table 4). The patient’s mother was also diagnosed with BBC and carried the mutation (Additional file 5: Figure S3). This mutation is located in the Tower domain of the protein and terminates the translation at the middle of OB2 domain as a result of which BRCA2 lacks its OB3 and NLC motifs at the C terminus. Similarly the second novel BRCA2 deletion located in exon 27 was observed in a patient BC24 with BBC (Table 4). The consequence of this mutation is a truncated protein without NLC2 and 3 motifs at the C terminus.

**Unclassified variants**

In addition to the unequivocally damaging mutations, many VUSs have been reported in index cases of high-risk BC/OC families in the absence of pathogenic mutation, and
their effect on the protein structure and function could not be immediately inferred [46]. Classifying these variants of unknown clinical significance as neutral or disease causing is very important for the genetic counseling.

We have identified in total 50 VUSs in both genes: 24 in BRCA1 and 26 in BRCA2, of which 24 were missense variants (Table 5). The in silico analysis suggested a possible deleterious effect of only five missense variants with all prediction programs (c.1067A > G, c.1648A > C and c.3113A > G in BRCA1 and 3515C > T and c.6100C > T in BRCA2) (Table 6). Another two: 139 T > G and c.536A > G in BRCA1 appeared to be clinically important according to POLYPHEN 2 and SIFT only (Table 6). One new BRCA1 missense variant c.3999 T > C was observed in a patient BC20 with BC and early onset in the absence of other pathogenic mutations, but the in silico analysis did not predict a possible pathogenic effect (Table 6).

The variant c.139 T > G that appeared to be clinically important upon in silico analysis is colocalized with the missense mutation c.181T > G in the RING finger domain of BRCA1 (exon 5) and has been recognized as one of the zinc-coordinating residues of the protein [10]. It was reported once in BIC database as a VUS [10]. Up to date there is no information available about its allele frequencies in different populations. Several functional studies have suggested a pathogenic effect of c.139T > G since in cell culture experiments it caused disability in coordination of the zinc ions in the RING domain and abolished the ubiquitin ligase activity of BRCA1 protein as well as its participation in the homologous recombination and the control of the centrosome number [25]. In our study the missense variant c.139T > G was found in one patient (0.5 %) with TNBC and early onset of the disease BC10, in the absence of other pathogenic mutation. However in order to ascertain its pathogenicity, further analysis of large genomic rearrangements in BRCA1/2 genes, as well as segregation analysis in the family of the patient need to be performed.

For all other missense variants that were predicted pathogenic by the in silico analysis (Table 6), the existing information in the literature has been controversial. For example, in some studies no association with BC has been found for the BRCA1 VUS c.1067A > G (Q356R), although the authors claimed that being homozygous for 356R might protect against BC [46]. According to other prediction models it demonstrated a possible harmful role [46]. Similar results have been obtained for the c.536A > G, c.1648A > C, c.3113A > G in BRCA1 and 3515C > T in BRCA2 [46]. Functional studies suggested that c.536A > G but not c.1648A > C variant might be related to BRCA1-associated pathogenicity by affecting its function in non-homologous end joining (NHEJ) [25].

In the present study c.536A > G and c.1648A > C in BRCA1, as well as c.3515C > T in BRCA2 (Table 5) were observed only in patients, with a low frequency (MAF < 0.005). In contrast 1067A > G and c.3113A > G in BRCA1 (Table 5) were detected in both patients and controls with a high frequency (MAF > 0.005).

The c.536A > G, c.1456 T > C and c.1648A > C have been often seen together and probably constitute a rare haplotype [47]. One of these substitutions, c.1456T > C was classified as neutral in the in silico analysis, in contrast to the other two (Table 6). We have observed this haplotype in one patient BC117 with family history of BC diagnosed at the age of 48. Her sister also developed BC at the age of 42, and subsequently died of the disease. We genotyped the patient’s healthy daughter and the two healthy daughters of her sister. One of the proband’s nieces was also carrier of the three missense variants. Identical case was reported in a Sicilian family where the proband with early onset of BC, her mother with early onset of uterine cancer and her healthy sister harboured the haplotype 536A > G, c.1456T > C, c.1648A > C [47]. It has been suggested that the three amino acid changes might alter the charge and stoichiometry of the protein and in consequence its function [47].

Even though the in silico analyses might assign deleterious function to some VUSs, they are not always consistent with the biological evidences. Further functional assays are necessary to prove the pathogenic effect of all missense variants predicted to be deleterious in current and previous studies.

Conclusions
As a result of the present study a mutation profile of the BRCA1/2 genes in Bulgarian BC/OC patients has been established with 13 (11 frameshift, 1 nonsense, 1 missense) unequivocally disease causing mutations. Mutations in BRCA1 gene were found in 14 % (28/200) and in BRCA2 in 5.5 % (11/200) of the Bulgarian patients selected by the recognized criteria for BRCA1/2 genetic testing. Four new frameshift (2 in BRCA1 and 2 in BRCA2 genes) have been found. Altogether inherited BC predisposition was identified in 39 (19.5 %) of the patients.

The most prevalent mutation in Eastern Europe c.5263_5264insC appeared to have a founder effect in the Bulgarian population with an overall frequency of 11 % in the studied cohort of familial BC/OC patients. It was also found in 14 % of the patients with TNBC without family history. Together with the other 3 recurrent mutations identified (c.181T > G in BRCA1; c.9098_9099insA and c.5851_5854delAGTT in BRCA2) they account for 77 % of all detected mutations. However, MLPA analysis is necessary to be performed in order to ascertain the contribution of the large genomic indels and rearrangements in BRCA1/2 genes for familial BC/OC development in the Bulgarian population.
Consequently, we suggest a mutation screening pipeline in which an initial test is performed to specifically detect BRCA1 c.5265_5264insC and the 3 additional recurrent mutations in BC patients from severely affected families to identify about two thirds of the carriers. In the remaining patients without mutations, complete sequencing of the coding regions of BRCA1 and BRCA2 is warranted, followed by MLPA screening. Such an approach would improve the effectiveness of the protocol and reduce the costs of mutation screening. This may have direct effect on the efficient molecular diagnostics of the genetic predisposition to BC/OC in Bulgaria.

Availability of supporting data
The data sets supporting the results of this article are included within the article and its additional files. The distribution of patients by criteria is represented in Additional file 1: Table S1. The list of primers sequences used for PCR amplification of the entire exons and exon-intron junctions of the BRCA1 and BRCA2 genes is shown in Additional file 2: Table S2. The pedigrees of the patients harboring the novel damaging mutations c.464delA and 5397_5403delCCCTTGG in BRCA1, and c.8532_8533delAA in BRCA2 are represented in Additional file 3: Figure S1, Additional file 4: Figure S2 and Additional file 5: Figure S3, respectively.

Additional files

Additional file 1: Table S1. Total number of BC patients distributed by criteria.

Additional file 2: Table S2. List of primers sequences used for PCR amplification of the entire exons and exon-intron junctions of the BRCA1 and BRCA2 genes.

Additional file 3: Figure S1. A pedigree of the patient BC134 harboring the novel damaging mutation c.464delA in BRCA1. The patient was diagnosed with TNBC at the age of 54 with three cases of BC in her family. Three of her healthy first degree relatives (sister, and 2 sons) were also carriers of the mutation.

Additional file 4: Figure S2. A pedigree of the breast cancer (BC) patient BC205 harboring the novel damaging mutation c.3975_4036delCCCTTGG in BRCA1. The patient was diagnosed with TNBC at the age of 51 and had a personal history of brain papilloma (BP) at the age of 18 and thyroid adenoma (TA) around 30 years of age. Her mother and great-grandmother were also diagnosed with BC at the age of 51 and 30, respectively. The healthy (HT) sister was screened for the presence of the mutation and was not a carrier (wt→wild type).

Additional file 5: Figure S3. A pedigree of the patient BC87 harboring the novel damaging mutation c.8532_8533delAA in BRCA2. The patient was diagnosed with bilateral breast cancer (BBC) and had early onset (y 30; y 37) of the disease. Similarly her mother was diagnosed with early onset BBC (y 37; y 38) and also carried the mutation.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
RD carried out the major part of the molecular genetic analysis, participated in the sequence alignment, data analysis, interpretation of the results and drafting of the manuscript; AM participated in the design and coordination of the study, sequence alignment, data analysis and interpretation of the results, and drafted the manuscript; DO participated in the molecular genetic analysis, sequence alignment, data analysis and interpretation of the results; LH, MC and SP participated in the molecular genetic analysis; AV, MT-H, SV, TD, TS, AI, KT, and SC recruited the patients and revised the clinical and pathological data; IP performed the in silico analysis of sequence variants and helped with the data analysis; IK participated in patient recruitment and genetic counseling; VM participated in the design and coordination of the study, and critical revision of the manuscript; RK participated in the design and coordination of the study, data analysis and interpretation of the results, helped with drafting and performed the final critical revision of the manuscript. All authors read and approved the final manuscript.

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