Identification of the most common BRCA alterations through analysis of germline mutation databases: Is droplet digital PCR an additional strategy for the assessment of such alterations in breast and ovarian cancer families?

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Abstract. Breast and ovarian cancer represent two of the most common tumor types in females worldwide. Over the years, several non-modifiable and modifiable risk factors have been associated with the onset and progression of these tumors, including age, reproductive factors, ethnicity, socioeconomic status and lifestyle factors, as well as family history and genetic factors. Of note, BRCA1 and BRCA2 are two tumor suppressor genes with a key role in DNA repair processes, whose mutations may induce genomic instability and increase the risk of cancer development. Specifically, females with a family history of breast or ovarian cancer harboring BRCA1/2 germline mutations have a 60-70% increased risk of developing breast cancer and a 15-40% increased risk for ovarian cancer. Different databases have collected the most frequent germline mutations affecting BRCA1/2. Through the analysis of such databases, it is possible to identify frequent hotspot mutations that may be analyzed with next-generation sequencing (NGS) and novel innovative strategies. In this context, NGS remains the gold standard method for the assessment of BRCA1/2 mutations, while novel techniques, including droplet digital PCR (ddPCR), may improve the sensitivity to identify such mutations in the hereditary forms of breast and ovarian cancer. On these bases, the present study aimed to provide an update of the current knowledge on the frequency of BRCA1/2 mutations and cancer susceptibility, focusing on the diagnostic potential of the most recent methods, such as ddPCR.

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Introduction

Breast and ovarian cancer are two of the most common malignancies affecting the female population both in industrialized and developing countries. According to GLOBOCAN 2020, breast cancer is the most frequently diagnosed tumor among females with 2,261,419 new cases (24.5%) and the first leading cause of cancer-related death with 684,996 deaths (15.5%). On the other hand, ovarian cancer represents the eighth most common cancer for incidence and mortality in females, accounting for 313,959 newly diagnosed cases (3,4%) and 207,252 deaths (4.7%) (1).

Over the years, several risk factors have been associated with the onset and progression of both breast and ovarian cancer (Fig. 1). As widely reported in the literature, more than half of all cases diagnosed are females aged >50 years, indicating that age is one of the major non-modifiable risk factors (2,3). Similarly, reproductive factors, such as age at menarche and menopause (before 11 and after 55 years of age, respectively), nulliparity and age at first full-term pregnancy (>35 years), are well-established risk factors for both breast and ovarian cancer (4,5). Furthermore, post-menopausal hormone therapies, based on the administration of estrogens plus progestin, significantly increase the risk of cancer development (6,7). Of note, the use of oral contraceptives for birth control has only been described as a risk factor for breast cancer, while oral contraceptive pills represent a protective factor for ovarian cancer, reducing the risk by at least 50% when used for 10 years or more (8-10). Other risk factors for both of these female cancer types include ethnicity, tobacco smoking, alcohol consumption, low physical activity, high-fat diet, obesity and socioeconomic status (11-16). Several studies

have demonstrated that environmental, lifestyle and epigenetic factors are associated with the development of both breast and ovarian cancer (17-20).

Besides the aforementioned modifiable and non-modifiable risk factors, a family history of breast and/or ovarian cancer, as well as genetic mutations, may have a key role in increasing cancer susceptibility. Of note, a growing body of evidence suggests that the risk of developing these female cancers is significantly increased in females having a first-degree relative affected by breast cancer or ovarian cancer or when the affected relative was under 50 years of age (21-24). Of note, over the years, several genetic mutations have been reported to be highly associated with an increased risk of both breast and ovarian cancer. Among these, BReast CAncer 1 (BRCA1) and BRCA2 are two tumor suppressor genes with high penetrance, which are involved in the activation of DNA repair processes and cell-cycle checkpoints in response to DNA damage (25,26). Functional deficiencies due to BRCA1/2 mutations induce genome instability, cell-cycle dysregulation and accumulation of other mutations (27,28). In this field, it has been widely demonstrated that BRCA1/2 mutations increase the lifetime risk to develop breast or ovarian cancer. Specifically, 5-10% of patients with breast cancer and 25% of ovarian cancer cases are due to an inherited genetic mutation affecting BRCA1 or BRCA2 (29,30). Furthermore, healthy individuals harboring germline mutations of BRCA1/2 had a 60-70% increased risk to develop breast cancer and a 15-40% increased risk for ovarian cancer (31,32). Therefore, the assessment of cancer-related risk factors, particularly the identification of BRCA1 and BRCA2 gene mutations, is crucial for the clinical management of females at a high risk of developing breast or ovarian cancer, which includes annual screening, chemoprevention and preventive surgery (33,34).

Although the impairment of DNA repair mechanisms due to BRCA mutations is associated with an increased risk of breast and ovarian cancer, patients developing these tumors benefit from therapies further affecting the DNA repair machinery aimed at killing cancer cells through the accumulation of several DNA alterations resulting in tumor cell death (35,36). Among these therapies, the use of poly [ADP-ribose] polymerase (PARP) inhibitors directed at PARP proteins involved in DNA repair mechanisms proved to be highly efficient in both ovarian and breast cancer (37,38).

The precise molecular characterization of both breast and ovarian tumors is essential to correctly classify cancer lesions and predict the prognosis of patients. With regard to breast cancer, the presence of hormone receptors (estrogen receptor and progesterone receptor), membrane receptors (epidermal growth factor receptor) and other molecular markers, including BRCA1/2 mutations, are used to classify breast cancer into different molecular subtypes (39). Besides its molecular classification, breast cancer may be divided according to histological features, e.g. ductal carcinoma, lobular carcinoma, mucinous carcinoma or spindle cell carcinoma (40,41).

As mentioned above, the precise identification of molecular markers such as BRCA1/2 mutations is essential to predict the prognosis of patients. In this context, both BRCA1 and BRCA2 mutations are associated with a higher aggressiveness of both breast and ovarian cancer (42). However, due to the development of novel targeted therapies using high-effective PARP inhibitors (olaparib, niraparib, recuparib and veliparib) for the treatment of tumors with BRCA mutations, the presence of these mutations in ovarian cancer is associated with a favorable prognosis (43). Despite the prognostic importance of BRCA1/2 mutations, the identification of other proteins or genetic and epigenetic factors is essential to predict the efficacy of treatments and the survival of patients (44).

The BRCA1 gene is located on the long arm of chromosome 17 (17q21) and it is composed of 24 exons (45). BRCA1 encodes for a multi-functional protein of 1,863 amino acids, which consists of an amino (N)-terminal RING domain, a carboxyl (C)-terminus, also known as the BRCT domain, and coding regions of exons 11-13 (Fig. 2) (46,47). These domains have a crucial role in the interaction between BRCA1 and several partner proteins. The RING domain (amino acids 1-109) is a highly conserved domain encoded by exons 2-7, which is characterized by a RING finger motif involved in the ubiquitination pathway. Of note, it heterodimerizes with BRCA1 associated RING domain 1 (BARD1) to form a dimeric RING ubiquitin-ligase (E3) (48). The BRCT domain is encoded by exons 16-24 and spans from amino acids 1,650-1,863, including two tandem repeats (~100 amino acids) linked by 22 amino acids. This domain binds to the phosphorylated serine-proline-x-phenylalanine motifs of different partner proteins, such as BTB domain and CNC homolog 1 (BACH1), BRCA1 interacting helicase 1, BRCA1 A complex subunit and C-terminal binding protein 1, to form functional macromolecular complexes that allow selecting the substrate for BRCA1-BARD1 activity (49-52). Compared to other domains, exons 11-13 cover a large part of the BRCA1 protein. Of note, exon 11 comprises two nuclear localization sequences (NLS) (amino acids 501-507 and 607-614), which facilitate the nuclear import process of BRCA1 interacting with importin α (53).

As widely described in the literature, BRCA1 may be considered a tumor suppressor gene whose derived protein is involved in several molecular pathways both in the nucleus and cytoplasm, including DNA double-strand break (DSB) repair, cell-cycle checkpoints, genome stability, transcription regulation, apoptosis, chromosomal segregation, mitochondrial genome repair, cytoskeletal rearrangements and centrosome regulation (Fig. 2) (54-57).

It has been reported that DNA DSBs activate several kinases, such as ATM, ATM-related kinase, checkpoint kinase 1 (Chk1) and Chk2, which phosphorylate BRCA1 (58,59). The hyperphosphorylated BRCA1 then interacts with several protein complexes that repair DSBs via homologous recombination repair (HRR) and the activation of cell-cycle checkpoints. Of note, BRCA1 is involved in HRR through the interaction with the RAD50-MRE11-NBS1 complex, as well as partner and localizer of BRCA2 (PALB2) and RAD51 DNA repair proteins (60-62). Regarding cell-cycle checkpoints, other BRCA1 complexes have been described. Specifically, G2/M checkpoint signaling is activated by BRCA1-receptor-associated protein 80, while the BRCA1-BACH1 complex is required during the S-phase (63,64). BRCA1 also regulates gene expression at the transcriptional level, interacting with RNA polymerase II and several transcription factors, including c-Myc, p53, histone deacetylase 1 and 2, signal transducer and activator

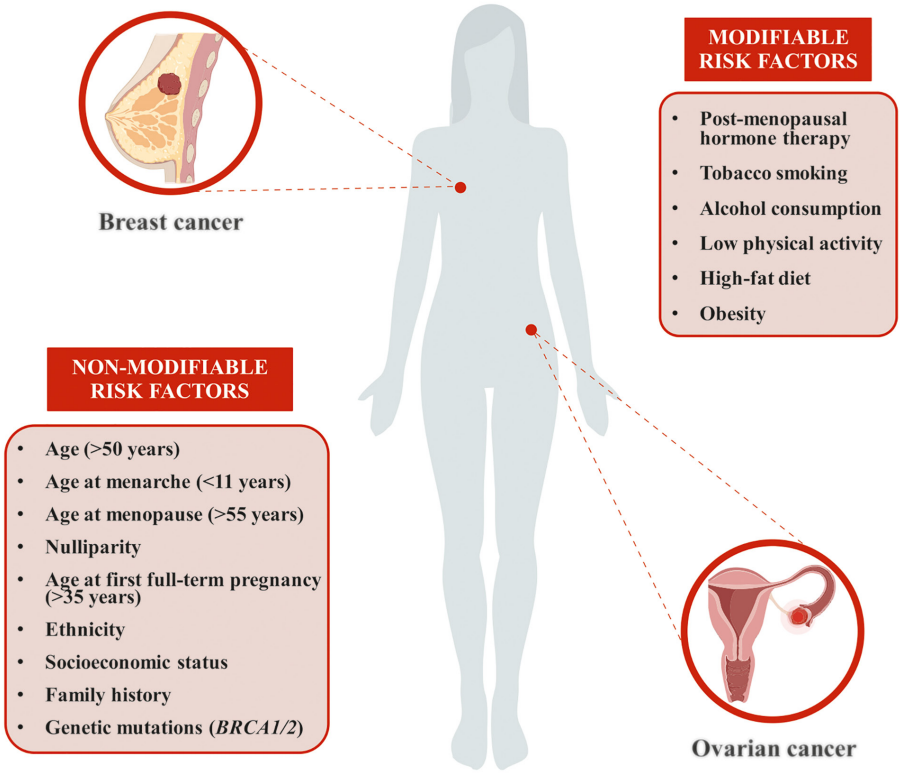

Figure 1. Non-modifiable and modifiable risk factors for breast and ovarian cancer.

of transcription 1 and zinc finger and BRCA1-interacting protein with KRAB domain-1, as well as the SWItch/sucrose non-fermentable complex (65-70). Of note, the ubiquitin-ligase activity of the BRCA1-BARD1 complex has a critical role in centrosome regulation. In particular, BRCA1 and BARD1 bind to Obg-Like ATPase 1, favoring the maintenance of centrosome numbers at S- and G2/M-phases (71). Furthermore, BRCA1 has apoptotic properties due to its nuclear export and the activation of the p53-independent growth arrest and DNA damage-inducible 45 regulatory sequences (72).

BRCA2 was described for the first time in 1995 by Wooster *et al* (73) analyzing breast cancer families. The BRCA2 gene is located on the long arm of chromosome 13 (13q12.3) and it is composed of 27 exons that encode for a protein of 3,418 amino acids. BRCA2 consists of an N-terminal domain, a middle region and a C-terminal domain, which mediate its interaction with different partner proteins (Fig. 3). Although the structures of BRCA1 and BRCA2 exhibit certain similarities, there is no sequence homology (74,75). Of note, the N-terminal domain, encoded by exons 1-10, spans from amino acids 1-636 and contains a transcription activation domain (TAD) essential for PALB2 binding (21-39 amino acids) (76). The central segment is encoded by exon 11 (637-2280 amino acids) and covers the major portion of the BRCA2 protein. This evolutionarily conserved region is characterized by eight repeats of ~35-40 amino acids, also known as BRC repeats, which represent the primary interaction sites for the recombination enzyme RAD51 (77). Regarding the C-terminal domain (exons 12-27), it spans from amino acids 2,281-3,418 and includes the DNA-binding domain composed of a helical domain (amino acids 2,482-2,668) and three oligonucleotide-binding folds (amino acids 2,670-3,184). Furthermore, the C-terminus of BRCA2 also includes two NLS, as well as an additional RAD51-binding site (TR2) (amino acids 3,270-3,305) (78).

Similar to BRCA1, the BRCA2 gene is considered a caretaker of genome stability that has a key role in several biological pathways. Of note, it has been reported that BRCA2 interacts with different partner proteins for the formation of macromolecular complexes performing distinct cellular functions, such as DNA DSBs repair by HRR, DNA replication fork stabilization, transcription regulation and cell cycle checkpoint regulation (Fig. 3) (79-82).

For instance, the interaction between BRCA2 and RAD51 is implicated in the repair of DNA damage by the HRR pathway. Of note, BRC repeats, as well as the C-terminal domain of BRCA2, regulate the assembly of RAD51 into a nucleoprotein filament, promoting strand invasion and the search for homologous DNA (83,84). Other partner proteins interact with BRCA2 forming macromolecular complexes that have a crucial role in DNA DSB repair and maintenance of genome stability, including BARD1, PDS5 cohesin-associated factor B and SEM1 26S proteasome subunit (85-87). BRCA2 also promotes the stabilization of stalled DNA replication forks. Specifically, BRCA2 directly interacts with PALB2 through TAD of the N-terminal domain to sustain the recruitment of polymerase η at blocked replication forks (88). Furthermore, Fanconi anemia complementation group D2 and biorientation of chromosomes in cell division 1-like proteins have been described to interact with BRCA2 and promote stalled fork protection (89,90). Of note, BRCA2 may act as a transcriptional co-regulator forming a functional complex with mothers against decapentaplegic homolog 3 (SMAD3). BRCA2 induces SMAD3-dependent transcriptional activation

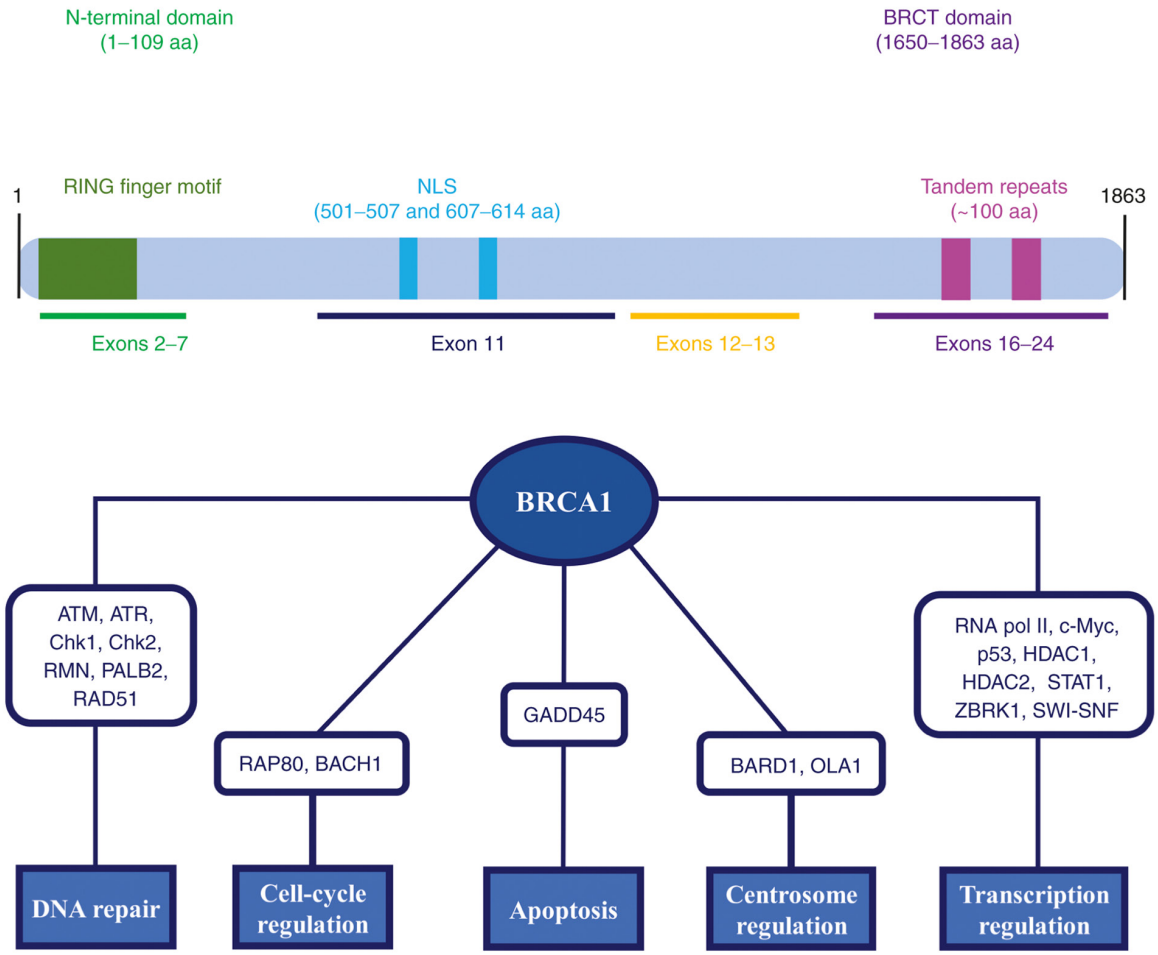

Figure 2. BRCA1 structure and functions. aa, amino acids; ATM, ataxia-telangiectasia mutated kinase; ATR, ATM-related kinase; BACH1, BTB domain and CNC homolog 1; BARD1, BRCA1 associated RING domain 1; BRCA1, BReast CAncer 1; BRCT, C-terminal domain; Chk1, checkpoint kinase 1; GADD45, growth arrest and DNA damage-inducible 45; HDAC1, histone deacetylase 1; NLS, nuclear localization sequences; OLA1, Obg-Like ATPase 1; RAP80, receptor-associated protein 80; RMN, RAD50-MRE11-NBS1 complex; PALB2, partner and localizer of BRCA2; RNA pol II, RNA polymerase II; STAT1, signal transducer and activator of transcription 1; SWI-SNF, SWItch/sucrose non-fermentable complex; ZBRK1, zinc finger and BRCA1-interacting protein with KRAB domain-1.

of plasminogen activator inhibitor 1, while SMAD3 increases the transcriptional activity of BRCA2, indicating a synergistic activity of these two proteins (91). Furthermore, it has been reported that the binding between BRCA2 exon 3 and the nuclear protein EMSY (BRCA2-interacting transcriptional repressor) is involved in chromatin remodeling and transcription regulation (92). Finally, although the direct role of BRCA2 in cell-cycle checkpoints remains to be fully clarified, the interaction between BRCA2 and BRCA2-associated factor 35 may be responsible for G2/M checkpoint modulation (93).

On these bases, the present study aimed to provide an update of the current knowledge on BRCA1 and BRCA2 mutations and cancer susceptibility, focusing on their physiological functions, mutation frequency and clinical impact, as well as the available genetic tests and new potential technologies for the detection of BRCA1/2 mutations.

Materials and methods

Identification of the most common BRCA1/2 genetic variants and their clinical significance. The analysis of BRCA1 and BRCA2 mutations was performed using the Breast Cancer Information Core (BIC) (https://research.nhgri.nih.gov/bic/, accessed on 3 February 2022), BRCA Exchange (https://brcaexchange.org/, accessed on 15 February 2022) and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/, accessed on 7 February 2022) public databases. Specifically, a total of 15,311 BRCA1 and 14,914 BRCA2 mutations were registered in the BIC database. Among these, the top 10 most common BRCA1 and BRCA2 genetic variants were selected for evaluation of their clinical significance. In this regard, the BIC designation of BRCA1/2 mutations was matched with data reported in the BRCA Exchange and ClinVar databases in order to classify them into variants that are benign, likely benign, pathogenetic or of uncertain significance.

Identification of BRCA1 and BRCA2 founder mutations. A literature search of studies published from 1995 until November 2021 was conducted using the PubMed public database (https://pubmed.ncbi.nlm.nih.gov/) in order to investigate interethnic mutation frequencies. The key words 'breast cancer', 'ovarian cancer', 'BRCA1 mutations', 'BRCA2 mutations', 'BRCA founder mutations', 'BRCA germline mutations' or 'BRCA somatic mutations' were used to identify potentially

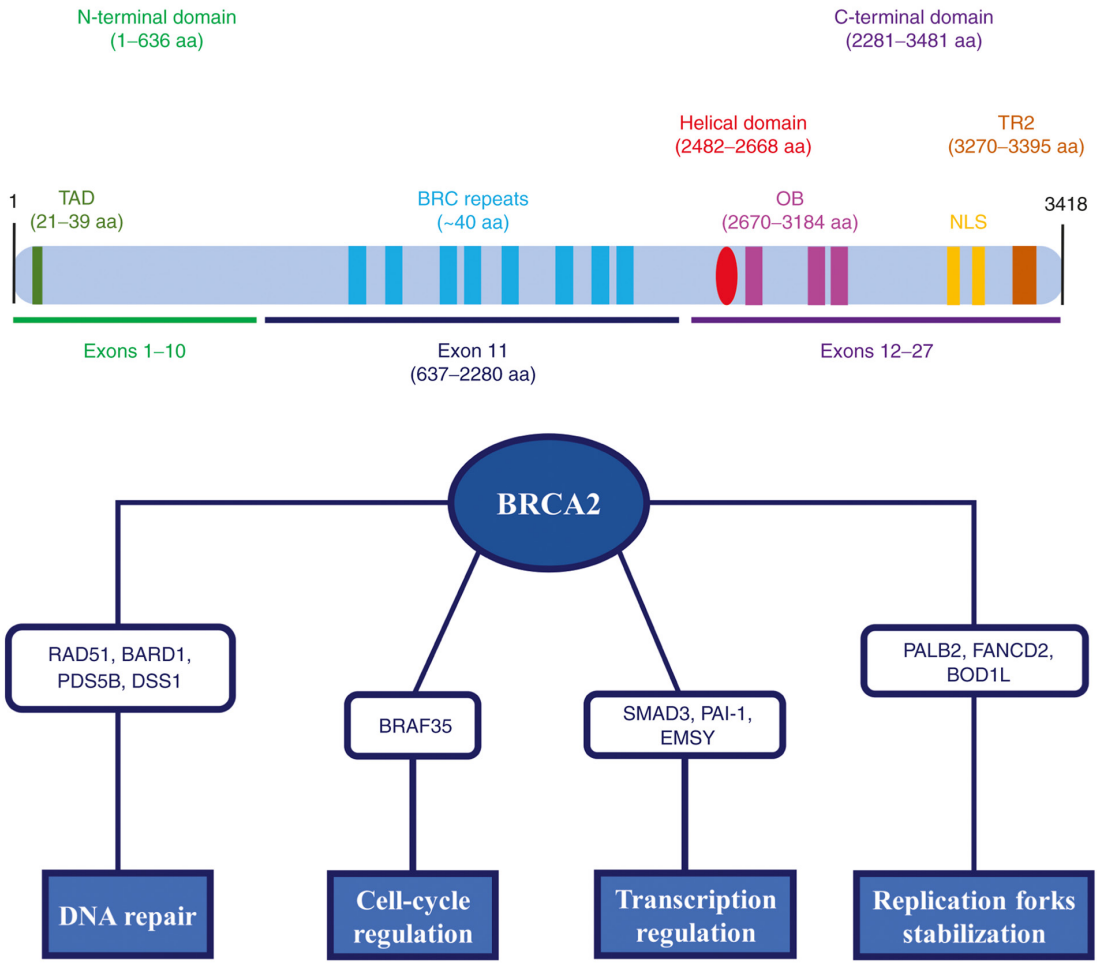

Figure 3. BRCA2 structure and functions. aa, amino acids; BARD1, BRCA1 associated RING domain 1; BOD1L, biorientation of chromosomes in cell division 1-like; BRAF35, BRCA2-associated factor 35; BRCA2, BReast CAncer 2; DSS1, SEM1 26S proteasome subunit; EMSY, BRCA2-interacting transcriptional repressor; FANCD2, Fanconi anemia complementation group D2; OB, oligonucleotide-binding; NLS, nuclear localization sequences; PAI-1, plasminogen activator inhibitor 1; PALB2, partner and localizer of BRCA2; PDS5B, PDS5 cohesin associated factor B; SMAD3, mothers against decapentaplegic homolog 3; TAD, transcription activation domain; TR2, RAD51-binding site.

relevant studies. In addition, the references contained in the most relevant studies were manually retrieved to find relevant articles not retrieved by PubMed exploration. Of note, the mutation frequency analysis was performed focusing on specific regions and ethnic groups, including Ashkenazi Jews, as well as populations from China, Denmark, Finland, France, Germany, Italy, Japan, Korea, Norway, Philippines, Poland, Russia and Sweden. In cases of overlapping data with the other published articles, the latest published and/or the larger sample size study was selected. Articles with ambiguous annotation of data, published in non-English or in Chinese language and duplicate publications were not included.

Results

BRCA1/2 mutation frequency and cancer susceptibility. Both endogenous and external DNA-damaging agents, including mutagens and radiation, as well as spontaneously occurring mutations, constantly threaten the integrity of the genome. Of note, DSBs represent the most damaging lesions of DNA, which may lead to chromosomal aberrations and mutations, increasing the risk of developing genetic disorders strictly related to cancer susceptibility (94). In this field, it has been widely reported that BRCA1 and BRCA2 tumor suppressor genes are involved in the repair of DNA DSBs by HRR. Specifically, BRCA1 promotes end resection and recruits PALB2, inducing chromatin localization of BRCA2. On the other hand, BRCA2 facilitates the recruitment of RAD51 recombinase, which inhibits the annealing of complementary ssDNA into the deleterious single strand (95). However, BRCA1 and BRCA2 mutations may increase the susceptibility to several tumor types, particularly breast and ovarian cancer (96,97). Indeed, BRCA1/2 loss of functions leads to genomic instability, which may result in the oncogenic transformation of normal cells into tumor-initiating cells (42).

BRCA1/2 mutations may be classified into germline and somatic mutations. Germline mutations are inherited in an autosomal dominant manner, while somatic mutations may arise *de novo* in tumor tissues due to a combination of genetic and environmental factors (98). Regarding BRCA1/2 germline mutations, loss of heterozygosity results in a non-functional protein that leads to Hereditary Breast and Ovarian Cancer (HBOC) syndrome, which is associated with increased susceptibility for these female tumors (99). Furthermore, inherited bi-allelic mutations of both BRCA1 and BRCA2 may cause congenital syndromes that are strictly associated with developmental

Table I. Top 10 most common BRCA1/2 mutations according to the BIC database.

BIC designation	Entries, n	Gene	HGVS nucleotide	HGVS protein	Mutation
185delAG	2038	BRCA1	c.68_69del	p.Glu23fs	Frameshift
5382insC	1093	BRCA1	c.5266dup	p.Gln1756fs	Frameshift
4427T>C	251	BRCA1	c.4308T>C	p.Ser1436=	Synonymous
S1613G	248	BRCA1	c.4837A>G	p.Ser1613Gly	Missense
C61G	239	BRCA1	c.181T>G	p.Cys61Gly	Missense
2430T>C	229	BRCA1	c.2311T>C	p.Leu771=	Synonymous
2201C>T	227	BRCA1	c.2082C>T	p.Ser694=	Synonymous
IVS18+66G>A	222	BRCA1	c.5152+66G>A	/	Intervening sequence
IVS16°68A>G	216	BRCA1	c.4987-68A>G	/	Intervening sequence
IVS16°92A>G	216	BRCA1	c.4987-92A>G	/	Intervening sequence
6174delT	1093	BRCA2	c.943T>A	p.Cys315Se	Frameshift
H372N	396	BRCA2	c.1114=	p.Asn372=	Missense
10°90A>C	346	BRCA2	c.*105A>C	/	3'UTR
F599S	345	BRCA2	c.1796=	p.Ser599=	Missense
IVS16-14T>C	332	BRCA2	c.7806-14T>C	/	Intervening sequence
IVS21-66T>C	319	BRCA2	c.8755-66T>C	/	Intervening sequence
K3326X	301	BRCA2	c.9976A>T	p.Lys3326Ter	Nonsense
I2490T	240	BRCA2	c.7469T>C	p.Ile2490Thr	Missense
3°24A>G	234	BRCA2	c.3396A>G	p.Lys1132=	Synonymous
IVS11+80delTTAA	221	BRCA2	c.6841+80_6841+83del	/	Intervening sequence

HGVS, Human Genome Variation Society; BRCA1, BReast CAncer 1; BIC, Breast Cancer Information Core.

anomalies, chromosomal fragility and increased cancer risk (100). In particular, the risk of breast cancer for females with a pathogenic BRCA1 or BRCA2 germline variant is 55-72% and 45-69%, respectively. Similarly, the risk for ovarian cancer is 39-44% for females with a BRCA1 germline variant and 11-17% for those with a BRCA2 germline variant (101). On the other hand, it has been reported that BRCA somatic mutations account for 15-30% of all BRCA1 and BRCA2 mutations. In addition, these non-inherited mutations are only present in 3% of all breast cancer cases (102).

Over the years, a large number of BRCA1/2 mutations have been described, and several of them are reliably known to increase cancer susceptibility (103). As reported in ClinVar, thousands of pathogenic or likely pathogenic BRCA1 and BRCA2 variants have been identified (>2,900 and >3,500, respectively) (https://www.ncbi.nlm.nih.gov/clinvar/, accessed 7 February 2022). The pathogenic or likely pathogenic mutations account for 80% of all mutations and result in a premature termination codon and truncated protein. Furthermore, missense mutations encoding a stable mutant protein account for 10% of all missense variants. Of note, frameshift mutations are more common in BRCA1, whereas missense mutations are more frequent in BRCA2 (104).

In the BIC database, a total of 15,311 BRCA1 mutations were registered, of which 6,133 are frameshift mutations, 4,577 are missense mutations and 1,421 are nonsense mutations. Regarding BRCA2, 14,914 mutations were registered (3,567 frameshift, 7,156 missense and 1,040 nonsense mutations). Of note, the mutation with the highest number of entries for BRCA1 was 185delAG, followed by 5382insC, whereas the most frequent mutation for BRCA2 was 6174delT (https://research.nhgri.nih.gov/bic/, accessed on 3 February 2022). Table I summarizes the most common BRCA1 and BRCA2 mutations registered in the BIC database.

Furthermore, according to the BRCA Exchange and ClinVar database, numerous BRCA1/2 mutations among the 10 most frequent in the BIC database are classified as benign or likely benign variants. Specifically, 4427T>C, S1613G, 2430T>C, 2201C>T, IVS18+66G>A, IVS16°68A>G and IVS16°92A>G are benign or likely benign BRCA1 variants, while 185delAG, 5382insC and C61G are certainly pathogenetic. Regarding BRCA2, 10°90A>C, IVS16-14T>C, IVS21-66T>C, K3326X, I2490T, 3°24A>G and IVS11+80delTTAA are registered as benign variants, H372N and F599S are variants with uncertain significance, and 6174delT is a pathogenic variant (https://brcaexchange.org/, accessed on 15 February 2022; https://www.ncbi.nlm.nih.gov/clinvar/, accessed on 7 February 2022).

BRCA1/2 founder mutations among the worldwide population. In the last decades, an increasing number of studies have reported that certain BRCA1/2 mutations, also known as founder mutations, were more frequent in specific regions and ethnic groups. For instance, 185delAG, 5382insC and 6174delT mutations have been detected in Ashkenazi Jews, which accounted for 99% of the pathogenic variants identified in this population. Of note, the 5382insC founder mutation has also been identified in other countries, such as Poland and Russia, accounting for 94 and 60% of BRCA1 mutations, respectively. Other BRCA1 founder mutations have been observed in these

Table II. BRCA1 founder mutations compared among different countries.

BIC Designation	HGVS nucleotide	Mutation	Population	(Refs.)
185delAG	c.68_69del	Frameshift	Ashkenazi Jewish	(106,107)
5382insC	c.5266dup	Frameshift	Ashkenazi Jewish	(106,107)
1100delAT	c.981_982del	Frameshift	China	(125)
2594delC	c.9613_9614delinsCT	Frameshift	Denmark	(114)
E1107X	c.3319G>T	Nonsense	Denmark	(114)
G1706A	c.5117G>C	Missmatch	Denmark	(114)
IVS1˚+3A>G	c.409˚+3A>G	Splicing variant	Finland	(113)
R1443X	c.4327C>T	Nonsense	Finland	(113)
3600del11	c.3481_3491del	Frameshift	France	(116)
G1710X	c.5128G>T	Nonsense	France	(116)
G1706A	c.5117G>C	Missense	Germany	(117)
C61G	c.181T>G	Missense	Germany	(117)
2804delAA	c.2685_2686del	Frameshift	Germany	(117)
IVS12-1643del3835	c.4186-1643_4357+2020del	Intervening sequence	Germany	(117)
5083del19	c.4964_4982del	Frameshift	Italy	(118-120)
L63X	c.188T>A	Nonsense	Japan	(121,122)
Q934X	c.2800C>T	Nonsense	Japan	(121,122)
1041del3insT	c.922_924delinsT	Nonsense	Korea	(123,124)
1675delA	c.1556del	Frameshift	Norway	(110-112)
816delGT	c.697_698del	Frameshift	Norway	(110-112)
3347delAG	c.3228_3229del	Frameshift	Norway	(110-112)
1135insA	c.1016dup	Frameshift	Norway	(110-112)
5454delC	c.5335del	Frameshift	Philippines	(126)
C61G	c.181T>G	Missense	Poland	(108,109)
4154delA	c.4035del	Frameshift	Poland	(108,109)
5382insC	c.5266dup	Frameshift	Poland	(108,109)
G1706A	c.5117G>C	Missense	Russia	(106,107)
5382insC	c.5266dup	Frameshift	Russia	(106,107)
3171ins5	c.3048_3052dup	Frameshift	Sweden	(115)

HGVS, Human Genome Variation Society; BRCA1, BReast CAncer 1; BIC, Breast Cancer Information Core.

countries, including C61G and 4154delA among the Polish population and G1706A in Russians (105-108).

Regarding the Northern European countries, the most common BRCA1 founder mutations detected among the Norwegian population were represented by 1675delA, 816delGT, 3347delAG and 1135insA (109-111). Furthermore, several BRCA1/2 mutations have been exclusively detected in individuals born in Finland, including IVS1˚+3A>G and R1443X for BRCA1 and IVS23+1G>A, 7708C>T and T8555G for BRCA2 (112). Of note, 2594delC, E1107X and G1706A represented the most common BRCA1 mutations in the Danish population, while 3171ins5 and 6601delA were detected among the Swedish (113,114).

As for Central European regions, it has been reported that 3600del11 and G1710X were the most frequently detected BRCA1 mutations in the French population (115). On the other hand, numerous founder mutations were detected among Germans, such as G1706A, C61G, 2804delAA and IVS12-1643del3835 for BRCA1 and 5579insA and 6503delTT for BRCA2 (116). Of note, the 5083del19 mutation exhibited a high rate in Italian families from Calabria, while 8765delAG was observed in certain Sardinian families (117-119).

Finally, different BRCA1/2 founder mutations have been also discovered in Asian populations. Of note, L63X, Q934X and 5802delAATT have been identified in Japanese, while the most common mutations among Koreans were 1041del3insT for BRCA1 and 7708C>T for BRCA2 (120-123). In addition, 1100delAT, 3337C>T and 9325insA were more frequently detected among Chinese, whereas 5454delC and 4859delA were the most common BRCA1/2 founder mutations in the Philippine population (124,125). The distribution of BRCA1/2 founder mutations is summarized in Tables II and III.

Discussion

To date, thousands of mutations have been identified in the BRCA1 and BRCA2 genes. Most of the described mutations are caused by small insertions or deletions, large genomic rearrangements (LGRs), as well as nonsense mutations and splice variants (126). As previously described, certain mutations have

Table III. BRCA2 founder mutations compared among different countries.

BIC designation	HGVS nucleotide	Mutation	Population	(Refs.)
6174delT	c.5946del	c.5946del	Ashkenazi Jewish	(106,107)
3337C>T	c.3109C>T	c.3109C>T	China	(125)
9325insA	c.9097dup	c.9097dup	China	(125)
IVS23+1G>A	c.9117+1G>A	Intervening sequence	Finland	(113)
7708C>T	c.7480C>T	Nonsense	Finland	(113)
T8555G	c.8327T>G	Nonsense	Finland	(113)
5579insA	c.5351dup	Frameshift	Germany	(117)
6503delTT	c.6275_6276del	Frameshift	Germany	(117)
8765delAG	c.8537_8538del	Frameshift	Italy	(118-120)
5802delAATT	/	Frameshift	Japan	(121,122)
7708C>T	c.7480C>T	Nonsense	Korea	(123,124)
4859delA	c.4631del	Frameshift	Philippines	(126)
6601delA	c.6373del	Frameshift	Sweden	(115)

HGVS, Human Genome Variation Society; BRCA2, BReast CAncer 2; BIC, Breast Cancer Information Core.

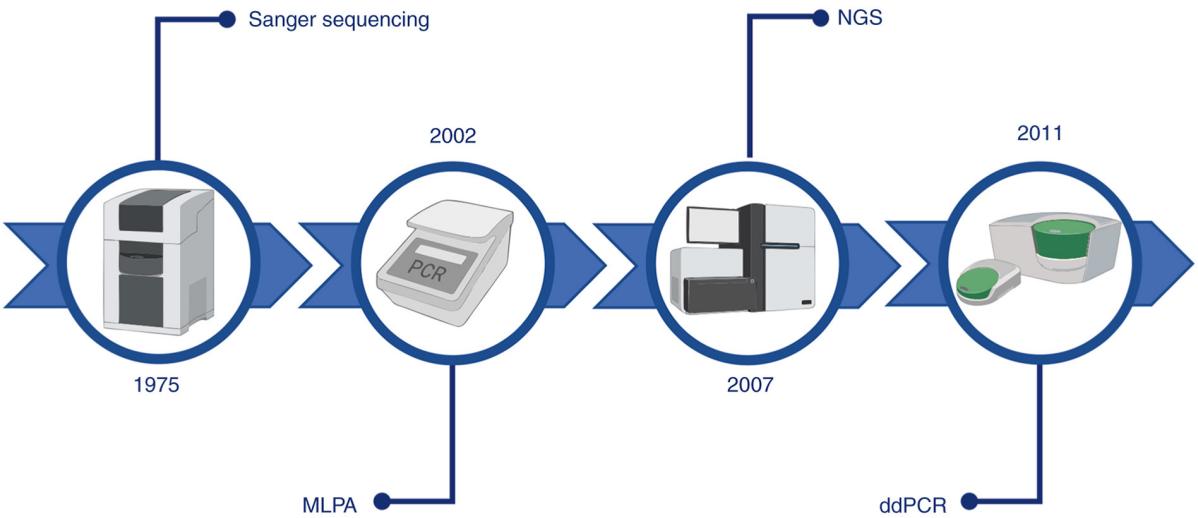

Figure 4. Schematic timeline of the technologies developed for the detection of BRCA1 and BRCA2 mutations. BRCA1, BReast CAncer 1; ddPCR, digital droplet PCR; MLPA, multiplex ligation-dependent probe amplification; NGS, next-generation sequencing.

been more frequently detected in specific geographical areas and ethnicities (127). Furthermore, it has been reported that each family group may carry a specific mutation that may be considered unique (128). Functional deficiencies due to these pathogenic mutations increase the lifetime risk to develop both breast and ovarian cancer. Of note, the most common cause of these female tumors is HBOC syndrome. Females harboring BRCA1/2 germline mutations have a higher risk of cancer development compared to other subjects (129,130). Therefore, the usage of reliable genetic tests for the assessment of BRCA1/2 mutations in high-risk females is crucial for the clinical management of patients.

Over the years, different platforms have been developed for the detection of BRCA mutations (Fig. 4). Among these, the Sanger method has been widely employed for genomic DNA sequencing. In brief, this method allows division of large genomic DNA into small fragments that are sequenced separately (131,132). Although the Sanger sequencing method is considered a reliable technology with a relatively simple workflow, it has several limits, such as sequencing throughput (a single DNA fragment at a time), time-consuming analysis and low cost-effectiveness (133).

Currently, the gold standard genetic test for the identification of BRCA1/2 mutations is next-generation sequencing (NGS). NGS is a high-throughput technology based on synthesis by sequencing millions of DNA fragments at once. Compared to Sanger sequencing, NGS has numerous other advantages, including automated analysis, faster turnaround time and higher sensitivity to detect LGRs and low-frequency variants with deep sequencing (134,135). However, NGS technology may also have certain disadvantages, such as complex workflow, high rates of variants with uncertain significance, higher cost and the reduction of sensitivity for large insertions/deletions (>20 bp) (136,137). The currently used standard

protocol for the identification of BRCA1 and BRCA2 mutations includes comprehensive sequencing and the assessment of LGRs. Furthermore, a single-site target may also be analyzed for patients with a first-degree relative affected by BRCA1/2 mutation (138,139).

The high cost of these technologies has prompted researchers and clinicians to develop novel high-sensitivity and low-cost strategies for the detection of BRCA1/2 mutations. Among these, reliable results were obtained by using the droplet digital PCR (ddPCR) platform.

ddPCR has recently emerged as a reliable tool with high sensitivity and specificity. In brief, the ddPCR system is based on a water-oil emulsion of the reaction mixture, which consists of a DNA sample, ddPCR Mastermix, primers and probe in a final volume of 20 μl (140). This procedure allows division of the sample into ~20,000 droplets that are transferred into a 96-well PCR plate for amplification. Since the sample is fractioned into thousands of droplets, PCR amplification takes place in each droplet. A droplet reader then detects the positive/negative signal of droplets depending on their amplified target and fluorescence amplitude (141).

Of note, ddPCR represents a valuable alternative to standard methods for the analysis of different clinical samples, such as fresh tumor biopsies and formalin-fixed paraffin-embedded (FFPE) tissues, as well as liquid biopsy samples (peripheral blood, sputum, urine, cerebrospinal fluid, stool, pleural effusions and ascites fluid), overcoming the limits due to poor DNA quality (142). Over the years, the potential clinical application of ddPCR has been demonstrated for absolute allele quantification, viral load quantification, DNA methylation, DNA copy number variation, germline/somatic mutation detection, circulating mutation detection, analysis of microRNAs, long non-coding RNAs and gene rearrangements (143-147). In this field, a growing number of recent studies have focused on the ddPCR system as a promising tool for the identification of specific BRCA1/2 mutations.

Preobrazhenskaya *et al* (148) developed a ddPCR assay to investigate BRCA1 LGRs in blood-derived DNA samples of patients with breast cancer (n=141). Using ribonuclease P RNA component H1 as a reference gene and PCR primers covering the entire coding region of BRCA1, the researchers identified three cases with exon 8 deletion and one case with exons 5-7 deletion (148). Furthermore, they identified a total of four cases with exon 8 deletion in an additional cohort of patients with breast and ovarian cancer (720 and 184, respectively). Collectively, the ddPCR data exhibited high concordance with multiplex ligation-dependent probe amplification (MLPA), a standard method for the detection of LGRs, suggesting that ddPCR may be a valuable tool for the assessment of BRCA1 LGRs (149). Similarly, another study evaluated the sensitivity and specificity of ddPCR to detect BRCA1 rearrangements in patients with serous ovarian cancer (149). In brief, the authors performed a multiplex ddPCR assay dividing primers/probe for BRCA1 exons into 8 groups along with albumin or ribonuclease P/MRP subunit P30 as a reference gene and identified nine cases with different BRCA1 deletions (100% concordance with MLPA) (149). Of note, Khalique *et al* (150) provided a case report of high-grade serous ovarian cancer (HGSOC) with secondary somatic mutation of BRCA2 (c.5446_5449delCTAG, p.Ser1816Leu fs*23). Specifically, the study highlighted that the ddPCR assay detected a higher mutation frequency (9.4%) in FFPE tissue samples compared to the whole-exome sequencing genetic test (2.3%) (150). Recently, De Paolis *et al* (151) further investigated the specificity of ddPCR in a cohort of patients with HGSOC positive for BRCA1 LGRs (exons 2, 20 and 21). By using RPLP0 as a reference gene and specific primers/probe, the ddPCR assay confirmed the detection of BRCA1 LGRs in blood, FFPE and fresh frozen tissue samples with 100% specificity (151).

Although no studies have been performed on the use of ddPCR for the detection of specific BRCA mutations in relatives of BRCA-positive cancer patients, the use of ddPCR for the detection of already known mutations in familial clusters would reduce the higher costs related to the whole sequencing of both BRCA1 and BRCA2 genes. Indeed, at present, the cost of a single test for the detection of BRCA mutations is 172$, while Garcia and colleagues described that the analysis of gene mutations performed by ddPCR cost half as much as NGS or other methods (152,153).

Besides the cost-effectiveness, ddPCR may also be used for the detection of circulating BRCA1/2 mutations in liquid biopsy samples. Indeed, ddPCR was effectively used for the detection of circulating mutations in patients with ovarian cancer, demonstrating how the detection of both germline and somatic ctDNA mutations is a valuable complementary tool for the diagnosis of BRCA-positive tumors and to establish the effectiveness of PARP inhibitors and the prognosis of patients with ovarian cancer (154).

All of these data suggest that ddPCR may be used for the detection of already known BRCA1/2 mutations in familial clusters of patients with a diagnosis of hereditary breast or ovarian cancer.

In conclusion, BRCA1 and BRCA2 germline mutations are well-established risk factors for the onset of breast or ovarian cancer. Overall, the computational investigations performed in the present study suggested that the BRCA1/2 mutations with the highest number of entries are classified as certainly pathogenic variants, such as 185delAG and 5382insC for BRCA1 and 6174delT for BRCA2. Furthermore, several of these mutations, also known as founder mutations, appear to be more frequently detected in specific geographical regions and ethnic groups. In this context, the development of low-cost and reliable genetic tests is fundamental to improve the screening program for the identification of females with germline BRCA1/2 mutations and the management of relatives of patients with a known diagnosis of hereditary breast or ovarian cancer. Currently, NGS is the gold standard for the assessment of BRCA1/2 mutations. In this field, ddPCR has recently emerged as a highly sensitive and specific technique. According to the studies described in the present article, ddPCR may be considered a promising alternative strategy for the detection of BRCA1/2 pathogenetic mutations. However, further studies should be performed to validate the application of ddPCR in routine clinical practice and surveillance strategies for hereditary female tumors.

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Availability of data and materials

The data reported in the manuscript are available from the corresponding author on request. The original data examined in the study are publicly available and may be retrieved from the following sources: www.pubmed.com; https://www.ncbi.nlm.nih.gov/clinvar/; https://research.nhgri.nih.gov/bic/; and https://brcaexchange.org/.

Authors' contributions

ML and AS conceptualized the study. AL, RR, GG and SC wrote the original draft of the manuscript. ML, AS, GNZ, GC and LF provided critical revisions. AL and RR prepared the tables and figures, conducted the formal analysis and critically analyzed the literature. All authors contributed to manuscript revision and read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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