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PRECLINICAL STUDY

Implementation of next-generation sequencing for molecular diagnosis of hereditary breast and ovarian cancer highlights its genetic heterogeneity

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Abstract Molecular diagnosis of hereditary breast and ovarian cancer (HBOC) by standard methodologies has been limited to the *BRCA1* and *BRCA2* genes. With the recent development of new sequencing methodologies, the speed and efficiency of DNA testing have dramatically improved. The aim of this work was to validate the use of next-generation sequencing (NGS) for the detection of *BRCA1/BRCA2* point mutations in a diagnostic setting and to study the role of other genes associated with HBOC in Portuguese families. A cohort of 94 high-risk families was included in the study, and they were initially screened for the two common founder mutations with variant-specific methods. Fourteen index patients were shown to carry the Portuguese founder mutation *BRCA2* c.156_157insAlu, and the remaining 80 were analyzed in parallel by Sanger sequencing for the *BRCA1/BRCA2* genes and by NGS for a panel of 17 genes that have been described as involved in predisposition to breast and/or ovarian cancer. A total of 506 variants in the *BRCA1/BRCA2* genes were detected by both methodologies, with a 100 % concordance between them. This strategy allowed the detection of a total of 39 deleterious mutations in the 94 index patients, namely 10 in *BRCA1* (25.6 %), 21 in *BRCA2* (53.8 %), four in *PALB2* (10.3 %), two in *ATM* (5.1 %), one in *CHEK2* (2.6 %), and one in *TP53* (2.6 %), with 20.5 % of the deleterious mutations being found in genes other than *BRCA1/BRCA2*. These results demonstrate the efficiency of NGS for the detection of *BRCA1/BRCA2* point mutations and highlight the genetic heterogeneity of HBOC.

Keywords HBOC · *BRCA1* · *BRCA2* · Next-generation sequencing · Targeted sequencing

Introduction

More than 20 years have passed since the identification of the two major breast cancer susceptibility genes, *BRCA1* and *BRCA2* [1, 2]. The identification of pathogenic mutations in these two genes in families with multiple cases of early-onset breast cancer was at the time a major breakthrough in hereditary cancer genetics. In *BRCA1* and *BRCA2* mutation carriers, the cumulative risk at 70 years of developing breast cancer is estimated to be 60 and 55 %, respectively, whereas for ovarian cancer it is estimated to be 59 and 17 %, respectively [3]. Genetic testing of *BRCA1/BRCA2* has several clinical implications, especially for female carriers, who should be offered the option to undergo annual MRI screening and mammography, prophylactic mastectomy, and/or salpingo-oophorectomy [4]. In addition, *BRCA1/BRCA2* mutation carriers can now benefit from the use of targeted therapy with the recent approval of PARP inhibitors for the treatment of ovarian cancer [5]. However, the contribution of *BRCA1/BRCA2* pathogenic mutations to high-risk breast cancer families is

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only around 30 %, and can vary according to the population and the criteria for selection of patients with predisposition to breast and/or ovarian cancer [6]. In a recent study from our group, 28.9 % of the families with an a priori BRCAPRO mutation probability >10 % harbored deleterious mutations in these genes [7].

Until now, molecular diagnosis of hereditary breast and/or ovarian cancer (HBOC) has been based on the identification of mutations in *BRCA1/BRCA2* and is usually performed by Sanger sequencing or alternative screening methods that are labor-intensive, and have low throughput and high turnaround time. With the recent development of next-generation sequencing (NGS), the speed and efficiency of DNA testing have dramatically improved. At the same time, NGS allows the possibility to analyze not only *BRCA1/BRCA2,* but multiple other genes that have been described as conferring an increased risk for the development of breast or ovarian cancer and that can explain a fraction of *BRCA1/BRCA2*-negative families. Germline mutations in *TP53* (Li–Fraumeni syndrome) [8], *CDH1* (Hereditary diffuse gastric cancer) [9], *STK11* (Peutz-Jeghers syndrome) [10], and *PTEN* (Cowden syndrome) [11] predispose to a variety of different cancers, but have in common the fact that they confer a high risk of breast cancer. Additionally, *PALB2*, *ATM*, *CHEK2,* and *NBN* are considered moderate-risk breast cancer genes [12–15]. On the other hand, mutations in Lynch syndrome genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*), together with those in *BRIP1*, *RAD51C,* and *RAD51D*, are associated with an increased risk for the development of ovarian cancer [16–19]. However, knowledge on the penetrance and the clinical utility of germline mutations in many of these genes is still incomplete [20]. The aim of this work was to validate the use of NGS for the detection of mutations in the *BRCA1* and *BRCA2* genes in a diagnostic setting by performing parallel analysis by Sanger sequencing and NGS in a consecutive series of high-risk breast/ovarian cancer families, as well as to evaluate the genetic heterogeneity in this setting by analyzing a panel of 17 genes associated with predisposition to those diseases.

Methods

Patients

The study included a consecutive series of 94 patients referred to the Genetics Department of the Portuguese Oncology Institute of Porto (IPO Porto) with a family history of breast and/or ovarian cancer and with either an a priori >20 % probability of finding a *BRCA1/BRCA2* mutation using the BRCAPRO software or a high-risk familial history for which BRCAPRO could underestimate the mutation probability. Samples for genetic testing were obtained after genetic counseling according to institutional review board approved guidelines and standard clinical practice. DNA was extracted from peripheral blood leucocytes, and its quality was evaluated using Qubit® Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

BRCA1/BRCA2 analysis

Screening of the Portuguese founder mutations (*BRCA1* c.3331_3334del and *BRCA2* c.156_157insAlu) was initially performed in all cases using a methodology we previously described [7]. In the 80 samples in which no founder mutations were identified, Sanger sequencing of the entire coding regions and adjacent intronic regions of *BRCA1* and *BRCA2* was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit in a 3500 Genetic Analyzer (Thermo Fisher Scientific), according to the manufacturer's instructions. Sanger sequencing was also performed for confirmation of all the deleterious variants identified by NGS. Multiplex Ligation-dependent Probe Amplification (MLPA) (MRC-Holland, Amsterdam, Netherlands) was used to detect *BRCA1/BRCA2* large genomic rearrangements (LGRs) in the 80 samples negative for founder mutations, according to the manufacturer's instructions.

Next-generation sequencing

Panel gene testing with NGS was used in the 80 samples, in which no founder mutations were found after the initial screening. Library preparation was performed using the TruSight Cancer kit (Illumina, Inc., San Diego, CA, USA), which targets the full coding sequence of 94 genes involved in hereditary predisposition to cancer, following the manufacturer's protocol. Sequencing was carried out using a standard flow cell in the MiSeq platform (Illumina, Inc.) in 2×150 bp paired end runs of 24 samples. Sequencing alignment and variant analysis were performed using a bioinformatics pipeline previously validated by us for 23 different genes (Paulo et al., submitted). In brief, alignment and variant calling were done using three different software programs, namely Isaac Enrichment (v2.1, Illumina, Inc.), BWA Enrichment (v2.1, Illumina, Inc.), and NextGENe (v2.3.4.4, Softgenetics, State College, PA, USA), with *.vcf* files being imported into GeneticistAssistant™ (Softgenetics) for variant annotation. For the purpose of this study, a virtual panel of 17 genes associated with predisposition to breast and/or ovarian cancer was created for variant analysis (Table 1). Variants were retained according to the following criteria: ≤10 % frequency in our in-house database, coverage ≥15x, alternative variant frequency ≥15 %, and minor allele frequency

Table 1 Genes included in the NGS panel associated with predisposition to breast/ovarian cancer

Gene	Reference sequence	Cancer risk	Median coverage
ATM	NM_000051.3	Breast	420
BRCA1	NM_007294.3	Breast/Ovarian	285
BRCA2	NM_000059.3	Breast/Ovarian	367
BRIP1	NM_032043.2	Ovarian	363
CDH1	NM_004360.3	Breast	315
CHEK2	NM_007194.3	Breast	303
MLH1	NM_000249.3	Ovarian	320
MSH2	NM_000251.2	Ovarian	380
MSH6	NM_000179.2	Ovarian	327
NBN	NM_002485.4	Breast	383
PALB2	NM_024675.3	Breast	324
PMS2	NM_000535.5	Ovarian	383
PTEN	NM_000314.4	Breast	370
RAD51C	NM_058216.2	Ovarian	339
RAD51D	NM_002878.3	Ovarian	255
STK11	NM_000455.4	Breast	161
TP53	NM_000546.5	Breast	242

(MAF) <1 %, excluding intronic variants more than 12 bp away from exon–intron boundaries. For MAF filtering, data were obtained from the 1000 Genomes Project (1000G; Phase III Data), Exome Variant Server (ESP6500), and Exome Aggregation Consortium (ExAC) databases.

Variant classification

Variants were classified as deleterious if they were predicted to originate a premature codon stop, if they were located in canonical splice sites, or if there were literature and/or own evidence to support their classification as pathogenic/likely pathogenic. The potential pathogenicity of the remaining variants, after variant filtering settings were applied, was evaluated depending on the type of mutation. Missense variants were evaluated using MetaSVM and MetaLR scores, which combine 10 different in silico prediction tools (SIFT, PolyPhen-2 HDIV, PolyPhen-2 HVAR, GERP++, MutationTaster, Mutation Assessor, FATHMM, LRT, SiPhy and PhyloP) and the maximum frequency observed in 1000G, having a higher predictive power than any of the prediction tools alone [21]. They were also evaluated using the Combined Annotation-Dependent Depletion (CADD) method, which integrates many diverse annotations into a single measure (C-Score) [22]. Missense variants were retained as variants of uncertain significance (VUS) only if they were predicted to be damaging by MetaSVM (rankscore >0.834), MetaLR (rankscore >0.823), and CADD (C-Score >15). Synonymous and intronic variants were retained only if they were predicted to have an impact on splicing by having at least a 15 % decrease in MaxEntScan and a 5 % decrease of the SpliceSiteFinder score, which was shown to have a 96 % sensitivity and 83 % specificity for the prediction of *BRCA1/BRCA2* VUS that result in a splicing defect when compared with transcript analysis [23]. Ada and RF scores (dbscSNV), two ensemble learning methods integrating several in silico prediction tools, were also evaluated with a cutoff value of 0.6 used [24]. In-frame deletions and insertions were also retained.

Results

Deleterious mutations in *BRCA1* and *BRCA2*

The two most common *BRCA1/BRCA2* mutations in the Portuguese population were screened in the 94 index patients under study, and 14 (14.9 %) were shown to be carriers of the *BRCA2* c.156_157insAlu (no *BRCA1* c.3331_334del carriers were identified). In the 80 samples negative for founder mutations, *BRCA1/BRCA2* screening of the entire coding regions was performed by Sanger sequencing. A total of 10 pathogenic mutations in *BRCA1* and seven in *BRCA2* were additionally detected, corresponding to a total of 31 (33 %) *BRCA1/BRCA2* pathogenic mutations identified in the 94 index cases analyzed. Personal and family cancer histories of all *BRCA1/BRCA2* carriers are detailed in Table 2.

In order to compare the efficiency of NGS for the detection of *BRCA1/BRCA2* point mutations, we analyzed the same 80 samples that were fully screened by Sanger sequencing using the TruSight Cancer panel. The comparison between NGS and Sanger sequencing was extended to all single-nucleotide variants (SNVs) and indels identified. Analysis was restricted to all the variants detected in the coding regions and 12 bp flanking the exons. All the variants detected by NGS with coverage ≤15× and alternative variant frequency ≤15 % were filtered out. A total of 506 variants (495 SNVs, 11 indels) were detected by NGS, giving a 100 % concordance with Sanger sequencing for detecting *BRCA1/BRCA2* point mutations (data not shown). A median coverage of 285 was obtained for *BRCA1* and of 367 for *BRCA2* (Table 1). Overall, 3840 regions were analyzed in both genes considering all samples, with only 33 (0.86 %) having at least one nucleotide with a coverage below 30 and 10 (0.26 %) with a coverage below 20 (data not shown).

Deleterious mutations in other genes

In the 80 samples where NGS was performed, we evaluated 15 other genes besides *BRCA1/BRCA2* that have been

Table 2 Deleterious mutations identified in the 80 index patients by NGS

Sample	Gene	HGVSc	Predicted protein	Personal history	Family history[a]
S25	*BRCA1*	c.211A>G	r.(spl?)	BC (34)	4× PrCa
S76	*BRCA1*	c.470_471del	p.(Ser157Ter)	OC (46)	2× BC
S75	*BRCA1*	c.2037delinsCC	p.(Lys679AsnfsTer4)	BC (47)	1× BBC, 1x PrCa
S63	*BRCA1*	c.2309C>A	p.(Ser770Ter)	BBC (34,34)	1× BC
S41	*BRCA1*	c.2418del	p.(Ala807HisfsTer8)	OC (46)	4× BC
S32	*BRCA1*	c.3477_3480del	p.(Ile1159MetfsTer50)	OC (41), BC (52)	–
S21	*BRCA1*	c.3817C>T	p.(Gln1273Ter)	BC (38)	1×BC
S44	*BRCA1*	c.3817C>T	p.(Gln1273Ter)	BC (40)	2× BC
S58	*BRCA1*	c.4165_4166del	p.(Ser1389Ter)	BBC (32,47)	3× BC, 1× PrCa
S49	*BRCA1*	c.5266dup	p.(Gln1756ProfsTer74)	BC (37)	3× BC
S54	*BRCA2*	c.2T>G	p.Met1?	BC (41)	4× BC
S61	*BRCA2*	c.793+1G>A	r.spl?	BC (49)	3× BC, 1× OC
S34	*BRCA2*	c.5934dup	p.(Ser1979Ter)	BC (52)	1× MBC
S52	*BRCA2*	c.6656C>G	p.(Ser2219Ter)	BC (60)	3× BC, 1× MBC
S55	*BRCA2*	c.7738C>T	p.(Gln2580Ter)	BC (50)	2× BC, 1× OC
S61	*BRCA2*	c.9097dup	p.(Thr3033AsnfsTer11)	BC (43)	1× BBC, 3× BC, 1× OC
S57	*BRCA2*	c.9453del	p.(Glu3152ArgfsTer11)	BC (50)	3× BC, 1× PrCa
S66	*PALB2*	c.1192del	p.(Val398CysfsTer26)	BC (52)	5× BC
S49	*PALB2*	c.1240C>T	p.(Arg414Ter)	BC (37)	3× BC
S67	*PALB2*	c.1633G>T	p.(Glu545Ter)	BC (47)	5× BC
S56	*PALB2*	c.2257C>T	p.(Arg753Ter)	BC (49)	1× BBC, 2x BC
S5	*ATM*	c.652C>T	p.(Gln218Ter)	BBC (36,48)	3× BC
S28	*ATM*	c.8264_8268del	p.(Tyr2755CysfsTer12)	CRC (57), BC (79)	1× BBC, 4× BC
S1	*CHEK2*	c.349A>G	p.(Arg117Gly)	BC (79)	1× BBC, 1× BC, 1× OC
S13	*TP53*	c.388C>T	p.(Leu130Phe)	CRC (17)	8× BC

BC breast cancer; *BBC* bilateral breast cancer; *OC* ovarian cancer; *PrCa* prostate cancer; *MBC* male breast cancer; *CRC* colorectal cancer

[a] Only tumors associated with HBOC were included: Breast, Ovarian, Prostate, and Pancreatic cancer

associated with increased risk of developing either breast or ovarian cancer. The median coverage ranged from 161 in *STK11* to 420 in *ATM* (Table 1). Deleterious mutations were detected in eight different families (10 %), four in *PALB2* (three nonsense and one frame-shift) (Fig. 1), two in *ATM* (one nonsense and one frame-shift) (Fig. 2), one missense mutation in *CHEK2* (Fig. 3a), and one missense mutation in *TP53* (Fig. 3b). The *CHEK2* missense mutation c.349A>G (p.Arg117Gly) has been reported in ClinVar as likely pathogenic, with functional studies showing that this variant results in a CHEK2 protein with impaired function due to reduced kinase activity, reduced protein stability, and incomplete phosphorylation [25–27]. The c.388C>T (p.Leu130Phe) missense mutation in *TP53* has been previously described as deleterious [28, 29]. Personal and family cancer histories of all carriers are detailed in Table 2.

Incidental findings

We detected an in-frame deletion of 15 bp in the *MSH6* gene (c.3848_3862del, p.Ile1283_Tyr1287del) in a patient diagnosed with breast cancer at the age of 32 years. This variant had been previously identified in two Lynch syndrome families in our laboratory with loss of MSH6 expression in the tumor (unpublished data), and it is also described as a causal mutation in the UMD database (www.umd.be) in a patient with colorectal cancer and loss of MSH6 expression in the tumor; hence, we consider it to be likely pathogenic. However, we did not observe loss of MSH6 expression in the breast tumor of our index patient (data not shown). Her family history includes an uncle diagnosed with male breast cancer at the age of 60 and both the maternal and paternal grandmothers diagnosed with colorectal cancer at the age of 72 (Online Resources 1).

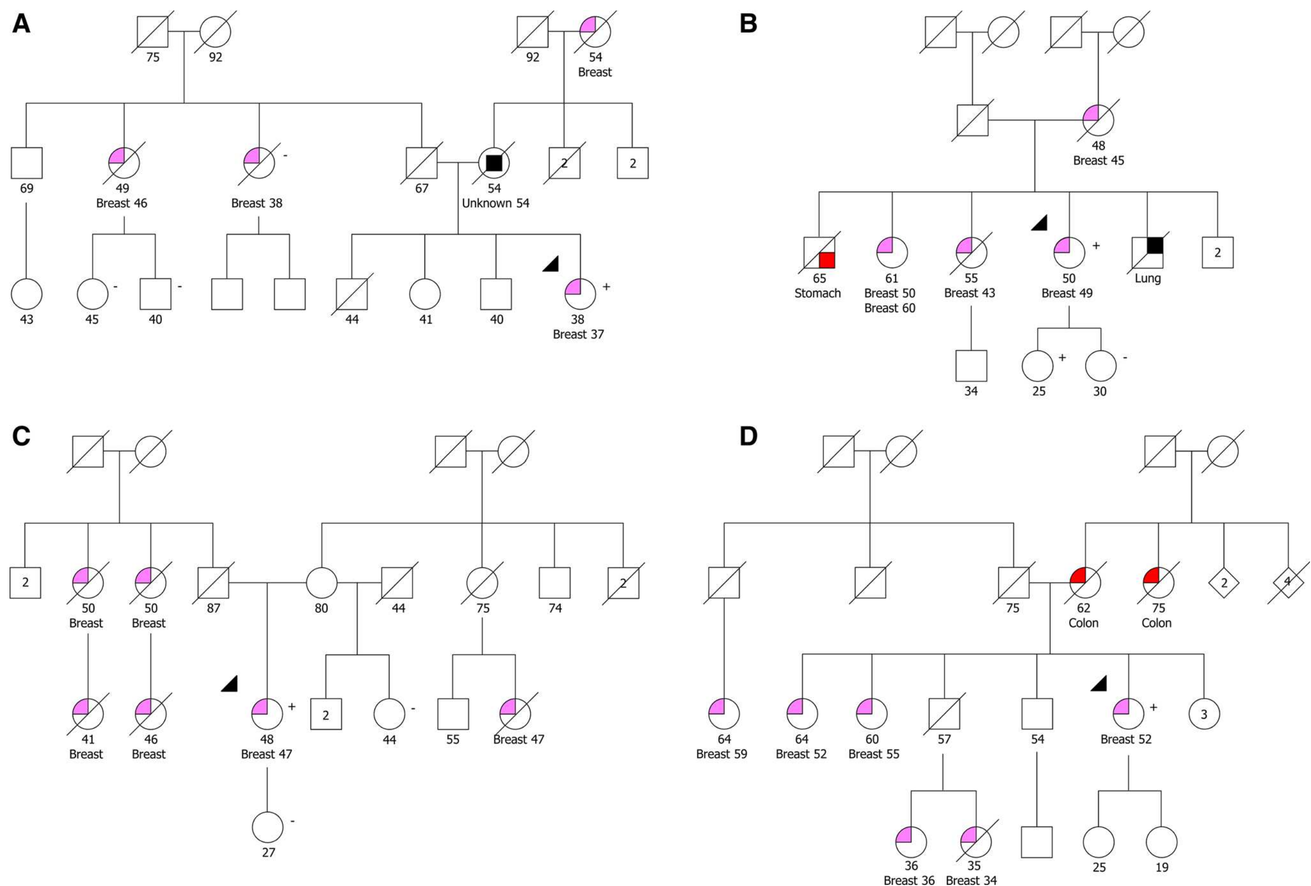

Fig. 1 Pedigrees of individuals with *PALB2* deleterious mutations. Family of the individual with both the *BRCA1* c.5266dup and the *PALB2* c.1240C>T mutation (**a**), the individual with the *PALB2* c.1633G>T mutation (**b**), the individual with the *PALB2* c.1192del mutation (**c**), and the individual with the *PALB2* c.2257C>T mutation (**d**). The index case is indicated by an *arrow*

Variants of uncertain significance

Applying the thresholds for missense and potential splicing mutations described earlier (see variant classification) after variant filtering, 10 missense variants were predicted to be deleterious, one variant was predicted to induce a splicing defect, and one in-frame deletion was retained (Table 3). Of these, eight variants (66.7 %) were observed in families where no clearly deleterious mutations were identified.

Discussion

NGS is increasingly being adopted in diagnostic laboratories because it offers higher throughput, faster turnaround time, and the possibility to expand the molecular diagnosis to rarer causative mutations, all without an increase in the cost of the analysis when compared to conventional methodologies. Nevertheless, before integration of NGS in a clinical setting, the efficiency of the methodology needs to be validated by individual laboratories, considering the different library preparation methods, the different sequencing chemistries, and especially the different bioinformatics algorithms for alignment, variant calling, and variant filtering available. We have recently established a bioinformatics NGS pipeline validated on a series of samples with various types of mutations in 23 different genes involved in hereditary predisposition to cancer (Paulo et al., submitted). In this study, we wanted to validate this previously established pipeline for the detection of *BRCA1/BRCA2* point mutations in a large series of high-risk HBOC patients and to take advantage of the higher throughput offered by NGS to characterize the involvement of other genes associated with an increased risk for developing breast and/or ovarian cancer.

We obtained 100 % sensitivity and specificity (total of 506 variants) for the detection of *BRCA1/BRCA2* point mutations with our bioinformatics pipeline using a targeted enrichment approach when compared to the gold standard Sanger sequencing. Although the majority of the variants were SNVs, 11 indels were present in the samples analyzed, which are known to be particularly sensitive to false negatives by NGS (Paulo et al. submitted) [30, 31]. Other studies have reported the validation of NGS for the

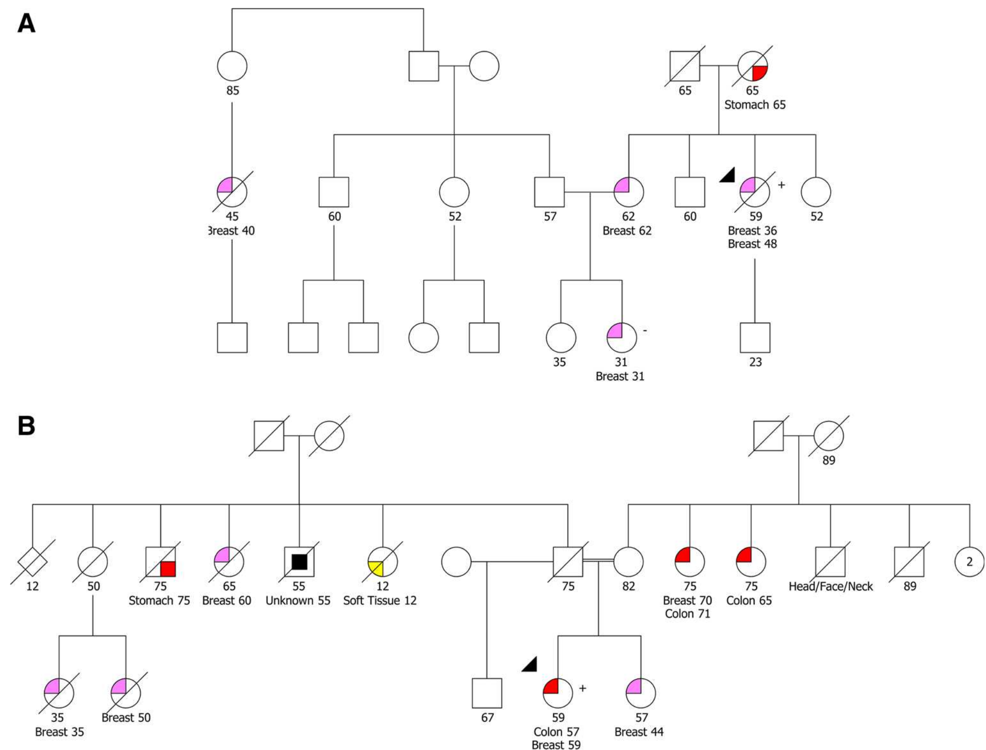

Fig. 2 Pedigrees of individuals with *ATM* deleterious mutations. Family of the individual with the *ATM* c.652C>T mutation (**a**) and the individual with the *ATM* c.8264_8268del mutation (**b**). The index case is indicated by an *arrow*

detection of *BRCA1/BRCA2* mutations using different workflows and platforms. All achieved a sensitivity of 100 % with false positives ranging from 1–1.8 % in Illumina platforms [32, 33] to 7.5–8.8 % on the Ion Torrent [31, 34]. In a diagnostic setting, low coverage regions require Sanger sequencing to ensure that a putative mutation is not missed because there were not enough reads covering that nucleotide. In our series, only 0.41 (33/80) or 0.13 (10/80) sequencing reactions per sample would be required if the minimum coverage threshold used was 30 or 20, respectively. Currently, molecular diagnosis of *BRCA1/BRCA2* needs to be completed by other methodologies, such as MLPA, for the detection of LGRs, but it is expected that in the future these will also be reliably detected by NGS with the validation of specific algorithms for detection of copy number variations, such as CONTRA, CNV-seq, or ExomeCNV [35–37].

A frequency of 33 % pathogenic *BRCA1/BRCA2* mutations was observed in our 94 patients, which is slightly higher than the frequency of 28.9 % that we previously observed in a larger series of HBOC patients [7], a difference that may be explained by the more stringent criteria used for cohort selection in the current study. The *BRCA2* c.156_157insAlu rearrangement remains the most frequent *BRCA1/BRCA2* mutation in our population (45 %), and this Alu insertion is not detectable using regular NGS bioinformatic algorithms designed for the detection of SNVs and indels [32] or by standard Sanger sequencing. Although its high frequency in our population warrants initial screening of this mutation before *BRCA1/BRCA2* full screening, in other populations patients with Portuguese ancestry should be offered specific testing for this mutation somewhere in the genetic testing algorithm [38]. Of all the other deleterious mutations identified in this study, the *BRCA2* c.2T > G deserves some attention, as it had been previously identified by our group and classified as a VUS due to nonsegregation in an affected relative in the initial family [39]. However, recent evidence suggests that mutations disrupting *BRCA2* initiation codon induce exon 2 skipping, with translation being initiated mostly at an out-of-frame ATG, leading to loss of protein function [40].

The other objective of this work was to characterize the spectrum of mutations in other genes predisposing to breast/ovarian cancer in high-risk families. We found deleterious mutations in eight families (10 % of the families analyzed by NGS and 8.5 % of all families), corresponding to 20.5 % of all deleterious mutations identified (8/39) (Fig. 4). In families negative for *BRCA1/BRCA2* mutations, the frequency of deleterious mutations was 11.1 % (7/63), which highlights the genetic heterogeneity underlying inherited predisposition to breast/ovarian cancer. Mutations were observed in *PALB2* (4), *ATM* (2), *CHEK2* (1), and *TP53* (1). *PALB2* mutations have been consistently described in familial and early-onset breast cancer, and the cumulative risk until age the age of 70 for developing breast cancer in a large cohort of *PALB2* mutation carriers has been reported to range from 33 %

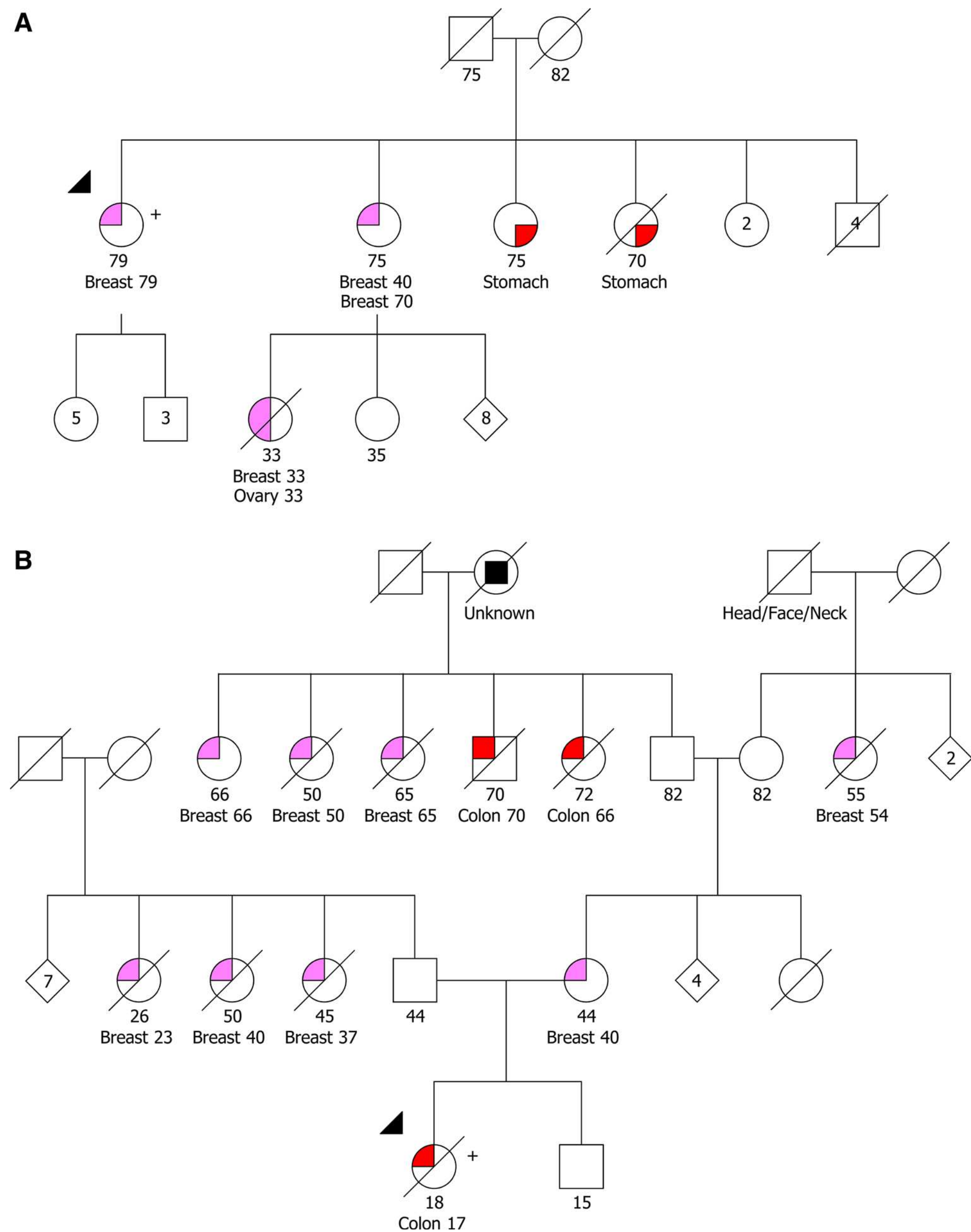

Fig. 3 Pedigrees of individuals with *CHEK2* and *TP53* deleterious mutations. Family of the individual with the *CHEK2* c.349A>G mutation (**a**) and the individual with the *TP53* c.388C>T mutation (**b**). The index case is indicated by an *arrow*

without family history taken into account to 58 % in those with a strong family history (being 44 and 67 %, respectively, at the age of 80), which is similar to the risks described for *BRCA2* [12]. In our study, mutations in this gene were found in 5 % of the families analyzed by NGS. In one of the families, a *BRCA1* pathogenic mutation was also identified, but they could have arisen from different branches of the family as both have relatives affected with breast cancer, with segregation studies required to confirm this possibility (Fig. 1a). Truncating variants in *ATM* also confer an increased risk to breast cancer (relative risk = 2.8), which seems to be similar to *CHEK2* (relative risk = 3.0) but lower than *PALB2* (relative risk = 5.3) [20]. Both the probands with *ATM* and *CHEK2* deleterious mutations had a family history of breast and/or ovarian cancer, but other tumors, such as colorectal, stomach, and soft tissue, were also present (Figs. 2,3a). We also detected a missense mutation in *TP53* in a proband diagnosed with colorectal cancer at the age of 17 and a significant family history of breast and colon cancer (Lynch syndrome had been excluded). Interestingly, this family did not fulfill the Chompret (or other) criteria for *TP53* mutation testing to diagnose Li–Fraumeni syndrome [29], being a good example of the potential of NGS to increase the molecular diagnosis yield in situations in which different syndromes have overlapping clinical features and in which genetic testing criteria do not have a 100 % sensitivity. Although the index patient had early-onset colorectal cancer, which

Table 3 Variants of uncertain significance identified in the 80 index patients by NGS

Sample	Gene	HGVSc	Predicted Protein	dbSNP ID	1000G_AF	ExAC_AF	ESP6500_AF	MetaSVM[a]	MetaLR[a]	CADD (C-Score)[a]	MaxEntScan (% decrease)[b]	SpliceSiteFinder (% decrease)[b]	Ada score[b]	RF score[b]
S67	*ATM*	c.1049C>T	p.Ala350Val	rs375049090	N/A	N/A	0.008	0.853	0.845	27.8	N/A	N/A	N/A	N/A
S80	*BRCA1*	c.80+5G>C	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	−48.8 %	−13.9 %	0.998	0.876
S36	*BRCA1*	c.190T>A	p.Cys64Ser	N/A	N/A	N/A	N/A	0.968	0.998	25.1	N/A	N/A	N/A	N/A
S21	*BRCA2*	c.4933_4935del	p.Lys1645del	N/A	N/A	0.001	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
S77	*BRCA2*	c.7975A>G	p.Arg2659Gly	rs80359026	N/A	N/A	N/A	0.960	0.958	27.7	N/A	N/A	N/A	N/A
S79	*BRCA2*	c.9004G>A	p.Glu3002Lys	rs80359152	N/A	N/A	N/A	0.910	0.903	22.4	N/A	N/A	N/A	N/A
S9, S49	*BRIP1*	c.139C>G	p.Pro47Ala	rs28903098	N/A	0.024	0.023	0.836	0.829	24.1	N/A	N/A	N/A	N/A
S39	*CHEK2*	c.757A>G	p.Lys253Glu	N/A	N/A	N/A	N/A	0.912	0.899	17.1	N/A	N/A	N/A	N/A
S60	*CHEK2*	c.1169A>C	p.Tyr390Ser	rs200928781	N/A	0.004	N/A	0.944	0.915	28.7	N/A	N/A	N/A	N/A
S3	*MLH1*	c.649C>T	p.Arg217Cys	rs4986984	0.060	0.032	N/A	0.952	0.943	22.4	N/A	N/A	N/A	N/A
S43	*MLH1*	c.2066A>G	p.Gln689Arg	rs63750702	N/A	0.028	0.023	0.840	0.877	22.2	N/A	N/A	N/A	N/A
S63	*MSH6*	c.3478G>A	p.Val1160Ile	rs376799914	N/A	0.005	0.008	0.864	0.866	22.1	N/A	N/A	N/A	N/A

N/A not available/not applicable

[a] Missense variants were retained as VUS if they were predicted to be damaging by MetaSVM (rankscore >0.834), MetaLR (rankscore >0.823) and CADD (C-Score >15) [21, 22]

[b] Synonymous and intronic variants were retained if they had at least a 15 % decrease in MaxEntScan, a 5 % decrease of the SpliceSiteFinder score and an Ada and RF score higher than 0.6 [23, 24]

is not part of the most typical tumor spectrum of either HBOC or Li–Fraumeni syndrome, this family had been selected because of very strong family history of early-onset breast cancer (especially from the paternal side, Fig. 3b), and indeed, recent data show that *TP53* mutations are found in 6 % of females with breast cancer diagnosed before the age of 31 in the absence of other features indicative of Li–Fraumeni syndrome, especially if their tumors are HER2-positive [41]. Some of the other genes included in our study and in many commercial NGS panels for HBOC still require further evidence from larger studies to confirm the relative risks for developing cancer, which will be helpful in determining their clinical utility. One example is *BRIP1*, which was initially described as conferring an increased risk for breast cancer [42], but a recent study in a large cohort of patients found no association of truncating variants with breast cancer risk [43]. Having said that, the most recent NCCN guidelines already recommend breast MRI screening for carriers of *ATM*, *CHEK2,* and *PALB2* mutations (in addition to previously known breast cancer high-risk genes *BRCA1*, *BRCA2*, *TP53*, *CDH1*, *STK11,* and *PTEN*), and that the possibility of risk-reducing mastectomy should be discussed with *PALB2* carriers. Carriers of *BRIP1*, *RAD51C,* and *RAD51D* mutations, on the other hand, should consider the option of performing risk-reducing salpingo-oophorectomy according to the latest NCCN guidelines, in line with what was already recommended for *BRCA1/BRCA2* and Lynch syndrome carriers [4].

With the adoption of NGS, there is some concern about the identification of incidental findings, disease-causing variants in high-penetrance genes in patients without the associated phenotype. In this study, we detected a likely pathogenic mutation in *MSH6* (c.3848_3862del, p.Ile1283_Tyr1287del) in a patient with breast cancer without loss of MSH6 expression in the tumor, indicating that her breast carcinoma was not related with the *MSH6* germline mutation, contrarily to the existent evidence for its involvement in the pathogenesis of colorectal cancer in typical Lynch syndrome families. Taking into account the family history of the patient, there was no indication to perform genetic testing of mismatch repair (MMR) genes (Online Resources 1), but the carriers of this mutation in this family are still at risk of developing Lynch syndrome-associated neoplasias, and adequate surveillance has been offered to the patient and her relatives after genetic counseling.

The use of bioinformatic tools is mandatory in order to compensate for the increased risk of finding VUS when one increases the number of genes analyzed by NGS, especially in whole-genome and whole-exome studies [21, 44, 45]. In this study, we report the use of a panel of 94 genes with analysis restricted to the genes of interest

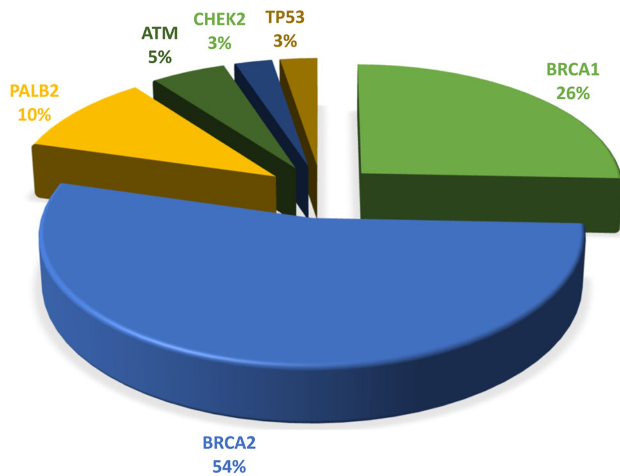

Fig. 4 Deleterious mutations identified per gene (%) in the 94 index patients

taking into account the clinical phenotype together with the use of in silico prediction tools for stratification of VUS. Although these tools cannot be used for classification of variants *per se*, they are useful for prioritization of VUS for further segregation and functional studies [23, 46]. We identified 12 VUS predicted to be deleterious in silico, eight of them in families where no clearly deleterious mutations were found, and these are the variants that we will prioritize for segregation studies (Table 3). The *BRCA1* c.190T > A (p.Cys64Ser) is located in the highly conserved RING domain of this gene, and there are already various missense mutations in this domain described as pathogenic [47, 48]. Other VUS were identified in *ATM, BRCA1, BRCA2, BRIP1, CHEK2, MLH1,* and *MSH6*, but the data available for these variants are scarce. Most of these variants may in the future be reclassified as deleterious or benign, but in the meantime they cannot be used to make clinical decisions.

There are some limitations in our study. Our sample size is relatively small, and we selected families with high risk to breast/ovarian cancer, which may increase the likelihood of identifying a deleterious mutation in breast/ovarian cancer-predisposing genes. Nonetheless, the frequency of *BRCA1/BRCA2* mutations identified is only slightly higher compared to a previous study where less stringent criteria were used, and it is not certain that mutations in moderate-penetrance genes are more likely to be found in high-risk families. Furthermore, the gene panel used in our study did not include the *RECQL* gene, which was recently reported to be associated with the risk of breast cancer in populations from Canada and Poland [49].

In conclusion, we have validated the use of NGS for the detection of *BRCA1/BRCA2* point mutations in a large series of patients, offering a higher throughput and higher molecular diagnostic yield in the study of inherited predisposition to breast/ovarian cancer and making possible to address its extensive genetic heterogeneity. This strategy allowed the identification of 39 deleterious mutations in 40 % of the families (38/94). The detection of deleterious mutations in some of these genes already has a significant impact in the clinical management of carriers, although further studies are necessary to make reliable estimates of cancer risk for many of the other genes included in current multigene panel testing to allow appropriate genetic counseling of these patients and their relatives.

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Compliance with ethical standards

Conflicts of interest The authors declare no conflicts of interest.

Ethical standards This study was performed according to the institutional review board approved guidelines and standard clinical practice, and informed consent was obtained from all individual participants included in the study.

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