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ARTICLE

Next-generation sequencing for the diagnosis of hereditary breast and ovarian cancer using genomic capture targeting multiple candidate genes

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To optimize the molecular diagnosis of hereditary breast and ovarian cancer (HBOC), we developed a next-generation sequencing (NGS)-based screening based on the capture of a panel of genes involved, or suspected to be involved in HBOC, on pooling of indexed DNA and on paired-end sequencing in an Illumina GAIIx platform, followed by confirmation by Sanger sequencing or MLPA/QMPSF. The bioinformatic pipeline included CASAVA, NextGENe, CNVseq and Alamut-HT. We validated this procedure by the analysis of 59 patients' DNAs harbouring SNVs, indels or large genomic rearrangements of *BRCA1* or *BRCA2*. We also conducted a blind study in 168 patients comparing NGS *versus* Sanger sequencing or MLPA analyses of *BRCA1* and *BRCA2*. All mutations detected by conventional procedures were detected by NGS. We then screened, using three different versions of the capture set, a large series of 708 consecutive patients. We detected in these patients 69 germline deleterious alterations within *BRCA1* and *BRCA2*, and 4 *TP53* mutations in 468 patients also tested for this gene. We also found 36 variations inducing either a premature codon stop or a splicing defect among other genes: 5/708 in *CHEK2,* 3/708 in *RAD51C*, 1/708 in *RAD50,* 7/708 in *PALB2*, 3/708 in *MRE11A*, 5/708 in *ATM*, 3/708 in *NBS1*, 1/708 in *CDH1*, 3/468 in *MSH2*, 2/468 in *PMS2*, 1/708 in *BARD1*, 1/468 in *PMS1* and 1/468 in *MLH3*. These results demonstrate the efficiency of NGS in performing molecular diagnosis of HBOC. Detection of mutations within other genes than *BRCA1* and *BRCA2* highlights the genetic heterogeneity of HBOC.
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INTRODUCTION

Since the last two decades, molecular diagnosis of hereditary breast and/or ovarian cancer (HBOC) is mostly based on the identification of germline inactivating mutations within *BRCA1* [MIM# 113705] and *BRCA2* [MIM# 600185].[1,2] In *BRCA1* and *BRCA2* mutation carriers, the cumulative risk of breast cancer at 70 years has been estimated to 65 and 45%, respectively, and the risk of ovarian cancer to 39 and 10%, respectively.[3] The identification of a deleterious *BRCA1/BRCA2* mutation within a family is crucial for the medical follow-up, as mutation carriers should be offered annual MRI or, alternatively, prophylactic mastectomy and prophylactic salpingo-oophorectomy. Furthermore, in a breast cancer patient, the detection of a germline *BRCA1* or *BRCA2* mutation may have important therapeutic consequences: complete mastectomy instead of partial mastectomy and, in the future, the prescription of specific targeted therapies, such as PARP inhibitors.[4,5] Considering the medical consequences of the identification of a germline *BRCA1* or *BRCA2* mutation and the frequency of mutation carriers, which has been estimated up to 1/500,[6] *BRCA1* and *BRCA2* are among the most frequently analyzed genes, in the context of Mendelian diseases. In molecular diagnostic laboratories, analysis of *BRCA1/BRCA2* is usually performed by Sanger sequencing, sometimes preceded by a pre-screening step mainly based on the detection of heteroduplexes using different methods such as denaturing high-performance liquid chromatography (DHPLC), high-resolution melting analysis (HRM) or enhanced mismatch mutation analysis (EMMA). This analysis is completed by the screening for genomic rearrangements, which is routinely performed using semi-quantitative methods, such as QMPSF or MLPA.[7–11] The number of amplicons and the need to combine several methods make the analysis of *BRCA1/BRCA2* particularly labour-intensive. Furthermore, screening for germline mutations within *BRCA1* and *BRCA2* often remains negative, even in families strongly suspected to present a Mendelian form of breast and/or ovarian cancer. Numerous other genes have been shown to be involved in the genetic determinism of breast or ovarian cancers, but their respective contribution and the penetrance of their mutations

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remain, for most of them, to be characterized. Mutations within *TP53*, *PTEN*, *STK11* and *CDH1* resulting in Li Fraumeni (LFS), Cowden, Peutz–Jeghers syndrome and hereditary diffuse gastric cancer, respectively, are associated to an increased risk of breast cancer;[12–15] mutations within *RAD51* paralogs, such as *RAD51C*, confer an increased risk of ovarian cancer;[16] and variations within *ATM*, *BRIP1*, *PALB2* and *CHEK2* have been shown to be associated with a moderate increase of breast cancer.[17]

Next-generation sequencing (NGS), based on massively parallel sequencing after clonal amplification of DNA templates in emulsion PCR or solid phase, allows molecular diagnostic laboratories to increase their throughput, to reduce the delay of analysis and to analyze simultaneously the different genes involved in a specific disease or a group of diseases. As recently highlighted, the main challenge of transferring NGS to medical diagnosis is the development of a workflow and bioinformatic pipelines fulfilling the requirement of quality control for diagnosis.[18] The aim of this study was to prospectively evaluate the performance of NGS for the routine analysis of *BRCA1* and *BRCA2* and to determine the rate of potential deleterious mutations within other genes in a large series of patients referred for a suspicion of HBOC. To fulfil both aims, we choose a strategy based on genomic capture and fully sequenced multiple genes, comparing three different capture designs.

PATIENTS AND METHODS

Patients

All the patients analyzed in this study have been seen in the context of a genetic session and fulfilled at least one the following criteria: (i) breast cancer before the age of 36, (ii) medullary breast adenocarcinoma before the age of 61, (iii) triple-negative breast cancer before the age of 41, (iv) male breast cancer before the age of 71, (v) ovarian adenocarcinoma before the age of 61, (vi) two breast cancer cases in first- or second-degree relatives (with a transmitting male), with at least one cancer before the age of 51 and the other before 71, (vii) three breast cancer cases in first- or second-degree relatives with at least one cancer before the age of 61, (viii) one breast cancer before the age of 51 in first-degree relatives with prostate cancer before the age of 61 or pancreas cancer before the age of 61.

A consecutive series of 708 patients was studied. Fifty-nine patients, previously genotyped for *BRCA1* and *BRCA2* and harbouring 62 representative variations, were used as controls (Table 1). For each patient, informed consent for genetic analysis was obtained.

Enrichment

We used Agilent eArray to design three different Sureselect solution library baits (Agilent, Santa Clara, CA, USA), covering a variable number of genes (Table 2). For each gene, exons and introns were covered by the capture. The first design had been kindly provided by MC King's laboratory and was used for the validation steps. Two different designs in addition to the first one were used to perform the prospective screening.[19]

Sample preparation and next-generation sequencing

DNA was extracted from peripheral blood, using the EZ1 BioRobot (Qiagen, Courtaboeuf, France). DNA was sonicated using a Covaris S2 (Covaris, Inc., MS, Woburn, MA, USA). The sample preparation was performed with SPRIworks System I or HT-High Throughput (Beckman, Villepinte, France). Illumina adapters were replaced by indexed adapters (Eurogentec, Angers, France), previously published by Huentelman's team.[20] The SureSelect enrichment process was performed either before combining indexed samples (hereafter called 'pooling after capture') according to the manufacturer's procedures (Agilent) or after combining equimolarly indexed samples (hereafter called 'pooling before capture') according to Kenny *et al.*[21] The current protocol, available on request, was robotized on two Biomek FX workstations dedicated to the pre- and post-PCR zone. Libraries were then sequenced on GAIIx (Illumina, San Diego, CA, USA) using the paired-end 2×76 bp program.

Bioinformatic analyses

The bioinformatic pipeline was automated using scripts in Python Programming Language and Java for creating report files (see Supplementary Figure S1). We used one Hash-based and one BWT-based aligner software. The CASAVA suite v1.8 from Illumina ensured demultiplexing, generation of .fastq files, mapping of the reads and variant calling, then the variants were annotated using Alamut-HT (Interactive BioSoftware, Rouen, France). The default setting of the Eland-pair analysis implemented in CASAVA was used with the 'variantsNoCovCutoff' option. In parallel, the NextGENe software v2.1 (SoftGenetics, State College, PA, USA) was used. NextGENe parameters were: single-end read analysis, 15 base seeds, 5 base move step, matching base percentage ≥ 70, 'detect large indels' option on, mutation percentage ≥ 10. The CNVseq software was implemented for the detection of genomic rearrangements within *BRCA1* and *BRCA2*.[22] A 500 bp 'windows size' parameter was set up and data were filtered with a log2 ratio ≥ 0.4 for duplication and ≤ -0.5 for deletion detection. For quality controls, bam files were inspected for coverage quality, using Samtools and the NextGENe software.[23] If the coverage was below $20\times$, the corresponding genomic region was checked by conventional methods (DHPLC-HRM-MLPA-Sanger sequencing). On *BRCA1* and *BRCA2*, the clinical significance of variations detected was based on consensus data integrated in the French UMD-*BRCA1/BRCA2* databases.[24] Variations detected within *TP53* were classified according the IARC *TP53* database.[25] For variations not listed as deleterious within the databases and for variations within the other genes of the capture, interpretations were based on the Align GV/GD tool and the minor allelic frequencies (MAF) estimated from the NHLBI GO Exome Sequencing Project Exome (ESP; http://evs.gs.washington.edu/EVS/).[26] In this study, we classified as potentially deleterious missense mutations with an AGVGD score $\geq$C45 and a ESP MAF < 0.01. Impact on splicing was predicted using MaxEntScan score and SpliceSiteFinder score. Variations outside the canonical AG/GT splice sites and inducing a 15% decrease of the MaxEntScan score and a 5% decrease of the SpliceSiteFinder score were considered potential splicing defects.[27] All mutations have been submitted to the Universal Mutation Database (http://www.umd.be/BRCA1/, http://www.umd.be/BRCA2/).

Confirmation of the detected variations

For *BRCA1* and *BRCA2*, all the SNV and indels detected using NGS within the coding sequence or ± 50 bp within the intronic sequences and not recorded as polymorphisms were confirmed by Sanger sequencing, using the BigDye Terminator Cycle Sequencing V1.1 Ready Reaction kit (Life Technologies, Carlsbad, CA, USA). Genomic rearrangements detected by CNVseq software were checked by using semi-quantitative methods, alternatively MLPA (MRC-Holland, Amsterdam, Netherlands) or QMPSF. For the other genes included in the capture enrichment, all variations inducing a premature codon stop or a potential splicing defect were checked by Sanger sequencing.

RESULTS

Validation of the NGS pipeline

First, we compared two indexing protocols based on pooling after capture and pooling before capture, respectively (Supplementary Figure S2). Twelve libraries prepared from patients harbouring known *BRCA1* or *BRCA2* mutations (patients T1 to T12, Table 1) were sequenced according to the two protocols, using the same index sequences. Each lane produced a mean number of reads equal to $60 \times 10^6 \pm 5 \times 10^6$ SD (Supplementary Figure S2). No obvious sequencing biases between indexes were noted after the demultiplexing procedure. The two protocols used did not show differences in quality sequencing parameters and in coverage. All the variations tested were correctly identified (Table 1). Then, using the pooling before capture protocol, we evaluated the NGS quality control

Table 1 ***BRCA1*** **and** ***BRCA2*** **variations used for the NGS validation**

Patients	*Gene*[a]	*Type*	*site*	*Description*[b]	*Expected consequence*[c]	*Detectable with Casava*	*Detectable with NextGene*	*Detectable with CNVseq*
T1	*BRCA1*	LGR	Del 1–24	c.1-?_5592+?del	p.?	NA	NA	Yes
T2	*BRCA1*	LGR	Dup 5–7	c.135-?_441+?dup	p.?	NA	NA	Yes
T3	*BRCA1*	indel	Ex 11	c.2612_2613insT	p.Phe872Valfs*31	Yes	Yes	NA
T4	*BRCA1*	LGR	Dup 13	c.4186-?_4357+?dup	p.?	NA	NA	Yes
T5	*BRCA1*	indel	Ex 13	c.4282ins39	p.Ser1428*	Yes	Yes	NA
T6	*BRCA1*	indel	Ex 18	c.5077_5080delinsTTCATTCTGC	p.Ala1693_Glu1694delinsPheIleLeuGln	Yes	Yes	NA
T7	*BRCA2*	indel	Ex 9	c.736_755del	p.Phe246Glnfs*2	Yes	Yes	NA
T8	*BRCA2*	indel	Ex 10	c.1231_1241delinsACAT	p.Ile411Thrfs*17	Yes	Yes	NA
T9	*BRCA2*	SNV	Ex 12	c.6848C>A	p.Pro2283His	Yes	Yes	NA
T10	*BRCA2*	indel	Ex 14	c.7069_7070del	p.Leu2357Valfs*2	Yes	Yes	NA
T11	*BRCA2*	indel	Ex 23	c.9097dup	p.Thr3033Asnfs*11	Yes	Yes	NA
T12	*BRCA1*	indel	Ex 11	c.1175_1214del	p.Leu392Glnfs*5	Yes	Yes	NA
T13	*BRCA1*	indel	Ex 11	c.3481_3491del	p.Glu1161Phefs*3	Yes	Yes	NA
T14	*BRCA1*	indel	Ex 11	c.3947_3950delTCTT	p.Phe1316*	Yes	Yes	NA
T15	*BRCA1*	indel	Int 20	c.5277+48_5277+59dup	p.?	No	Yes	NA
T16	*BRCA1*	indel	Ex 11	c.3731_3738del	p.His1244Argfs*8	Yes	Yes	NA
T17	*BRCA2*	indel	Int 8	c.681+97_681+98delGT	p.?	Yes	Yes	NA
T17	*BRCA1*	indel	Int 21	c.5332+182dup	p.?	Yes	Yes	NA
T18	*BRCA1*	indel	Ex 6	c.282_288dup	p.Thr97Alafs*2	Yes	Yes	NA
T19	*BRCA1*	indel	Ex 11	c.2709_2710del	p.Cys903*	Yes	Yes	NA
T20	*BRCA1*	indel	Ex 11	c.1016dup	p.Val340Glyfs*6	Yes	Yes	NA
T21	*BRCA1*	indel	Ex 11	c.3770_3771del	p.Glu1257Glyfs*9	Yes	Yes	NA
T22	*BRCA1*	indel	Ex 11	c.3541_3556del	p.Val1181Leufs*24	Yes	Yes	NA
T23	*BRCA1*	indel	Ex 14	c.4391_4393delinsTT	p.Pro1464Leufs*2	Yes	Yes	NA
T24	*BRCA1*	indel	Ex 11	c.2269del	p.Val757Phefs*8	Yes	Yes	NA
T25	*BRCA1*	indel	Ex 11	c.3839_3843delinsAGGC	p.Ser1280*	Yes	Yes	NA
T26	*BRCA2*	indel	Ex 25	c.9435_9436del	p.Ser3147Cysfs*2	Yes	Yes	NA
T27	*BRCA2*	indel	Ex 16	c.7795_7797del	p.Glu2599del	Yes	Yes	NA
T28	*BRCA2*	SNV	Ex 10	c.1395A>C	p.=	Yes	Yes	NA
T28	*BRCA2*	indel	Ex 10	c.1389_1390del	p.Val464Glyfs*3	Yes	Yes	NA
T29	*BRCA2*	indel	Ex 18	c.8053del	p.Thr2685Hisfs*9	Yes	Yes	NA
T30	*BRCA2*	indel	Ex 10	c.1796_1800del	p.Ser599*	Yes	Yes	NA
T31	*BRCA2*	indel	Ex 11	c.6408_6414del	p.Asn2137Lysfs*29	Yes	Yes	NA
T32	*BRCA2*	indel	Ex 8	c.635_636del	p.Arg212Lysfs*2	Yes	Yes	NA
T33	*BRCA2*	indel	Ex 11	c.6079dup	p.Arg2027Lysfs*22	Yes	Yes	NA
T34	*BRCA2*	indel	Ex 27	c.9699_9702del	p.Cys3233Trpfs*15	Yes	Yes	NA
T35	*BRCA2*	indel	Ex 9	c.755_758del	p.Asp252Valfs*24	Yes	Yes	NA
T36	*BRCA2*	indel	Ex 11	c.5810_5811del	p.Ser1937Trpfs*7	Yes	Yes	NA
T37	*BRCA2*	indel	Ex 22	c.8773_8780dup	p.Arg2927Serfs*3	Yes	Yes	NA
T38	*BRCA1*	SNV	Int 5	c.213-1G>A	p.?	Yes	Yes	NA
T39	*BRCA1*	SNV	Ex 11	c.3979C>T	p.Gln1327*	Yes	Yes	NA
T40	*BRCA2*	SNV	Int 6	c.516+2T>C	p.?	Yes	Yes	NA
T41	*BRCA2*	SNV	Ex 13	c.7007G>A	p.Arg2336His	Yes	Yes	NA
T42	*BRCA2*	SNV	Ex 12	c.6848C>A	p.Pro2283His	Yes	Yes	NA
T43	*BRCA2*	SNV	Ex 15	c.7558C>T	p.Arg2520*	Yes	Yes	NA
T44	*BRCA2*	SNV	Ex 21	c.8707G>T	p.Glu2903*	Yes	Yes	NA
T45	*BRCA2*	SNV	Ex 3	c.273C>A	p.Tyr91*	Yes	Yes	NA
T46	*BRCA1*	LGR	Del 1–2	c.-232-?_80+?del	p.?	NA	NA	Yes
T47	*BRCA1*	LGR	Del 11–12	c.671-?_4185+?del	p.?	NA	NA	Yes
T48	*BRCA1*	LGR	Del 3	c.81-?_134+?del	p.?	NA	NA	Yes
T49	*BRCA1*	LGR	Del 8–13	c.442-?_4357+?del	p.?	Yes	Yes	NA
T49	*BRCA2*	indel	Ex 11	c.5272_5274del	p.Asn1758del	Yes	Yes	NA
T50	*BRCA1*	LGR	Del 3–16	c.81-?_4986+?del	p.?	NA	NA	Yes
T51	*BRCA1*	LGR	Del 5–24	c.135-?_5592+?del	p.?	NA	NA	Yes
T52	*BRCA1*	LGR	Del 17	c.4987-?_5074+?del	p.?	NA	NA	Yes
T53	*BRCA1*	LGR	Dup 3–8	c.81-?_547+?dup	p.?	NA	NA	Yes
T54	*BRCA1*	LGR	Del 22	c.5333-?_5406+?del	p.?	NA	NA	Yes
T55	*BRCA1*	LGR	Del *NBR2*-2	c.-232-?_80+?del	p.?	NA	NA	Yes

Table 1 (Continued)

Patients	Gene[a]	Type	site	Description[b]	Expected consequence[c]	Detectable with Casava	Detectable with NextGene	Detectable with CNVseq
T56	*BRCA1*	LGR	Del 21	c.5278-?_5332+?del	p.?	NA	NA	Yes
T57	*BRCA1*	LGR	Del 20	c.5194-?_5277+?del	p.?	NA	NA	Yes
T58	*BRCA2*	LGR	Del 12–13	c.6842-?_7007+?del	p.?	NA	NA	Yes
T59	*BRCA2*	indel	Ex 3	c.156_157insAlu[d]	p.?	Yes	No	No

Abbreviations: LGR, large genomic rearrangement; NA, not applicable.
[a]Nomenclature was numbered on the basis of the transcript NM_007294 for *BRCA1* and NM_000059 for *BRCA2*.
[b]Mutation nomenclature according to HGVS recommendations, nucleotide position was numbered with +1 corresponding to the A of the ATG of the translation initiation codon.
[c]Expected consequence on the protein level.
[d]Corresponding to the Portuguese founder mutation c.156_157insAP003441.3:g.105088_105370.

parameters on 48 other control DNA samples (T6 and T13 to T59; Table 1) harbouring 430 SNV and indels, and 13 large genomic rearrangements within *BRCA1* or *BRCA2*. Each lane of GAIIx produced $90 \times 10^6 \pm 6 \times 10^6$ SD reads. The average on-target ratio was equal to $42 \pm 1\%$ SD. The coverage in targeted regions was on average equal to $225 \pm 27\times$ SD for six multiplexed libraries. Our NGS pipeline detected 438 SNV and indels, and 17 large genomic rearrangements. All the known variations tested were detected, showing a sensitivity of 100%. The eight additional detected SNV and indels were not confirmed by Sanger sequencing corresponding to 1.8% of false positives. Most of them were recurrent transversion variants with unbalanced forward/reverse strand ratio. Similarly, the four additional large genomic rearrangements were not validated by MLPA/QMPSF. Two discrepancies were noted between CASAVA and NextGENe software (Table 1). The *BRCA2* c.156_157insAP003441.3:g.105088_105370 also known as c.156_157insAlu in patient T59, corresponding to a Portuguese founder mutation,[28] was correctly detected only by CASAVA using orphan paired reads data and the *BRCA1* c.5277+48_5277+59dup mutation in patient 15 was identified only by NextGENe. These two discrepancies led us to keep in the bioinformatics pipeline both the CASAVA and the NextGENe software.

The last step of the validation study was conducted on a group of 168 consecutive previously not analyzed patients by performing, in parallel, NGS and DHPLC-HRM-MLPA-Sanger sequencing. These methods had been routinely used in our laboratories for the molecular diagnosis of HBOC, in more than 4000 patients. Excluding polymorphisms, 85 variations, including 14 causal variations, of which one was a genomic rearrangement, were detected within in *BRCA1* and *BRCA2*. All the variations detected by our conventional procedures were also detected by NGS.

Detection, using the NGS pipeline, of *BRCA1*, *BRCA2* and *TP53* mutations

The NGS workflow was applied to the molecular diagnosis of HBOC in 708 new patients, using different versions of the capture set (Table 2). A total of 69 germline deleterious mutations (37 in *BRCA1* and 32 in *BRCA*2) were detected (Table 3): 53 mutations were predicted to induce a premature termination codon (PTC), 10 were previously known to induce a splicing defect, 3 corresponded to genomic rearrangements and 3 were known deleterious missense mutations. Eight additional missense variations within *BRCA2* suspected to be deleterious were also detected. Among this series of patients (see the inclusion criteria in Materials and methods), the overall mutational detection rate on *BRCA1* and *BRCA2* was therefore 10.8%, which is comparable with the rates obtained by the French diagnostic laboratories (http://www.e-cancer.fr). We also detected four *TP53* germline mutations, recorded as deleterious: c.638G>C, p.Arg213Pro; c.646G>A, p.Val216Met; c.704A>G, p.Asn235Ser; c.1010G>A, p.Arg337His (according to the reference sequence NM_000546.5). Among the four corresponding families (Supplementary Figure S3), only one family clearly met the Chompret criteria for the LFS.[29] Three rare variants (EVS MAF <0.01) of unknown significance were also found in *TP53*: c.664C>T, p.Pro222Ser; c.1025G>A, p.Arg342Gln; c.1060C>A, p.Gln354Lys.

Detection, using the NGS pipeline, of mutations affecting other genes

In the other panel genes, 36 variations inducing a PTC or affecting the canonical AG/GT splice sites were detected and were classified as deleterious (Tables 2 and 4, Figure 1). These mutations accounted for one-third of the mutations classified as deleterious in our series (Figure 1). Among 468 families, deleterious mutations within the MMR genes *MSH2* and *PMS2* were detected in five distinct families, among which two included at least one relative with an ovarian cancer (Supplementary Figure S4). Only one deleterious mutation was found in *CDH1*, and retrospectively, gastric cancers were mentioned within the corresponding family (data not shown). We also detected, among 708 patients, 10 inactivating mutations within *PALB2* and *RAD51C* and 10 inactivating mutations within *CHEK2* and *ATM* (Table 4). In addition, according to the thresholds described in Materials and methods (section Bioinformatic analyses) for missense changes and for potential splicing mutations, 28 missense changes could be suspected to be deleterious and 11 variants were predicted to induce splicing defect (Table 4, Supplementary Table S1).

DISCUSSION

The transfer of NGS from research to diagnostic laboratories is today one of the most important challenges in medical genetics. If NGS offers considerable possibilities in terms of throughput, the entire procedure, including the bioinformatic analyses, should fulfil the quality requirement of diagnostic laboratories. Here, we show and validate the efficiency of the entire NGS procedure for the molecular diagnosis of HBOC. Indeed, all SNVs, indels and genomic rearrangements tested and previously detected by conventional methods, were detected using our pipeline. Compared with the classical methods commonly used for genetic analyses, the sensitivity of the entire procedure was estimated to be 100% and we observed 1.8% of false positives. We found, that the CNVseq algorithm was able to detect genomic rearrangements with a good sensitivity and specificity. Nevertheless, this algorithm is time-processor-consuming and,

Table 2 Description of the different capture designs and number of detected mutations

Targeted genes	*Design 1*	*Design 2*	*Design 3*	*Number of patients tested*	*Mutations inducing PTC*	*Splicing mutations*[a]	*Deleterious missense mutations*[b]	*Potential splicing mutations*[c]	*Potential deleterious missense mutations*[d]
BRCA1	x	x	x	708	28	3	3		
BRCA2	x	x	x	708	25	7			8
TP53	x		x	468			4		3[e]
ATM	x	x	x	708	4	1		1	9
BAP1		x	x	379					
BARD1	x	x	x	708	1				
BRIP1	x	x	x	708					
CDH1	x	x	x	708	1				
CHEK2	x	x	x	708	3	2		2	8
MLH1	x		x	468					2
MLH3	x		x	468	1			1	
MRE11A	x	x	x	708	3			1	1
MSH2	x		x	468	3			2	
MSH6	x		x	468					1
NBS1	x	x	x	708	3			4	1
PALB2	x	x	x	708	7				3
PMS1	x		x	468	1				1
PMS2	x		x	468	1	1			2
PTEN	x	x	x	708					
RAD50	x	x	x	708	1			1	
RAD51			x	139					
RAD51B			x	139				1	
RAD51C	x	x	x	708	2	1			
RAD51D		x	x	379					
STK11	x	x	x	708					
XRCC2			x	139					
XRCC3			x	139					

Abbreviation: PTC, premature termination codon.
[a]Mutations within the canonical AG/GT splice sites, or mutations previously known to induce a splicing defect in *BRCA1*, *BRCA2* and *TP53*.
[b]Published deleterious missense mutations.
[c]Variations within the consensus sites inducing a 15% of decrease of the MaxEntScan sore and a 5% decrease of the SpliceSiteFinder except mutations listed in [a].
[d]Missense mutations with an Align GVGD score >C45 and a MAF in ESP samples <0.01 excluding those in [b] and excluding published neutral variants.
[e]Missense mutations in *TP53* reported as 'probably deleterious' were only filtered against MAF in ESP samples <0.01.

therefore, other software such as CONTRA should offer an interesting alternative.[30] A major advantage of the NGS procedure is that the progressive implementation of specific software should allow the detection of other types of alterations, which are not found by using conventional procedures. For instance, complex rearrangements, such as inversions, should be detected taking advantage of paired-end data, by using software such as PINDEL.[31] Deep intronic mutations probably constitute a reservoir of undetected mutations and the capture of intronic sequences represents also an additional advantage of a capture-based NGS strategy in the future, when tools for the interpretation of intronic mutations will be available.

In terms of efficiency, transition from conventional to NGS procedures has allowed our medium-size molecular diagnostic laboratory (including three full time technicians, two bioinformaticians and two medical geneticists) to perform 1000 complete screenings of *BRCA1/BRCA2* per year, each analysis being completed and validated within a maximum of 3 months, in a routine procedure. We observed that the implementation of NGS did not result into a dramatic increase of reagent cost per patient, as this cost was evaluated, for the conventional and for the NGS procedures, to 292 and 311 euros, respectively. The main consequence, in terms of human power is the integration of fulltime bioinformaticians in medical diagnostic laboratories, regardless the type of platform.[32] In our experience, this is indeed absolutely crucial to construct the informatics pipeline, to evaluate the numerous available software and to generate quality reports at each step of the process.

Another advantage of a NGS procedure based on gene capture is the possibility to simultaneously analyze other genes involved in the phenotype. Therefore, an additional aim of our study was to estimate, on a large series of patients, the mutation detection rate within the other genes that have been demonstrated or suspected to be involved in HBOC. Among these, the other gene whose mutations also confer a high risk for breast cancer is *TP53*. Early-onset breast cancer is one of the canonical tumours of the LFS spectrum and the lifetime breast cancer risk of germline *TP53* mutations has been estimated to be 49%.[33] *TP53* mutation detection rate in women with breast cancer before 36 years of age and without detectable *BRCA1* or *BRCA2* mutation has been estimated to be 7%.[29,34] Our *TP53* mutation detection rate in this series of 468 patients analyzed for *TP53* was lower (~1%), which can easily be explained by the fact that our patients had not been selected on an early age of breast cancer onset. In patients harbouring germline *TP53* mutations, several studies have highlighted the risk of secondary tumours in the field of radiotherapy suggesting that, in a breast patient with germline *TP53* mutation, radiotherapy should be avoided.[35–37] This is a strong additional argument justifying the inclusion of *TP53* in a diagnostic NGS panel for breast cancer. Nevertheless, considering the wide LFS tumour spectrum and tumour risk in children, *TP53* testing should be

Table 3 Deleterious mutations found in *BRCA1* and *BRCA2* by NGS in 708 patients

Gene	*Transcript*	*HGVS nomenclature*	*Expected consequence on the protein*	*Mutation type*
BRCA1	NM_007294.3	c.181T>G	p.Cys61Gly	Missense
BRCA1	NM_007294.3	c.1421T>G	p.Leu474*	PTC
BRCA1	NM_007294.3	c.1444_1447del	p.Ile482*	PTC
BRCA1	NM_007294.3	c.1789G>T	p.Glu597*	PTC
BRCA1	NM_007294.3	c.1965C>A (*n*=2)	p.Tyr655*	PTC
BRCA1	NM_007294.3	c.2008G>T	p.Glu670*	PTC
BRCA1	NM_007294.3	c.211del	p.Arg71Glyfs*17	PTC
BRCA1	NM_007294.3	c.212+3A>G	p.?	Splicing mutation
BRCA1	NM_007294.3	c.2125_2126insA	p.Phe709Tyrfs*3	PTC
BRCA1	NM_007294.3	c.2359dup	p.Glu787Glyfs*3	PTC
BRCA1	NM_007294.3	c.2668del	p.Ser891Profs*2	PTC
BRCA1	NM_007294.3	c.2709_2710del	p.Cys903*	PTC
BRCA1	NM_007294.3	c.2722G>T	p.Glu908*	PTC
BRCA1	NM_007294.3	c.3018_3021del	p.His1006Glnfs*17	PTC
BRCA1	NM_007294.3	c.3228_3229del	p.Gly1077Alafs*8	PTC
BRCA1	NM_007294.3	c.3329del	p.Lys1110Serfs*7	PTC
BRCA1	NM_007294.3	c.3839_3843delinsAGGC (*n*=2)	p.Ser1280*	PTC
BRCA1	NM_007294.3	c.3841C>T	p.Gln1281*	PTC
BRCA1	NM_007294.3	c.4065_4068del	p.Asn1355Lysfs*10	PTC
BRCA1	NM_007294.3	c.4327C>T (*n*=2)	p.Arg1443*	PTC
BRCA1	NM_007294.3	c.4945_4947delinsTTTT	p.Arg1649Phefs*30	PTC
BRCA1	NM_007294.3	c.5075-2A>G	p.?	Splicing mutation
BRCA1	NM_007294.3	c.5095C>T	p.Arg1699Trp	Missense
BRCA1	NM_007294.3	c.5193+2delT	p.?	Splicing mutation
BRCA1	NM_007294.3	c.5266dup (*n*=4)	p.Gln1756Profs*74	PTC
BRCA1	NM_007294.3	c.5324T>G	p.Met1775Arg	Missense
BRCA1	NM_007294.3	c.5503C>T	p.Arg1835*	PTC
BRCA1	NM_007294.3	c.676del	p.Cys226Valfs*8	PTC
BRCA1	NM_007294.3	c.81-?_4986+?del	p.?	LGR
BRCA1	NM_007294.3	c.442-?_4357+?del (*n*=2)	p.?	LGR
BRCA2	NM_000059.3	c.1184G>A	p.Trp395*	PTC
BRCA2	NM_000059.3	c.1310_1313del	p.Lys437Ilefs*22	PTC
BRCA2	NM_000059.3	c.1593dup (*n*=3)	p.Glu532Argfs*3	PTC
BRCA2	NM_000059.3	c.1813del	p.Ile605Tyrfs*9	PTC
BRCA2	NM_000059.3	c.2627del	p.Asn876Ilefs*19	PTC
BRCA2	NM_000059.3	c.2701del	p.Ala902Leufs*2	PTC
BRCA2	NM_000059.3	c.2786dup	p.Leu929Phefs*7	PTC
BRCA2	NM_000059.3	c.2808_2811del	p.Ala938Profs*21	PTC
BRCA2	NM_000059.3	c.316+5G>C	p.?	Splicing mutation
BRCA2	NM_000059.3	c.4889C>G	p.Ser1630*	PTC
BRCA2	NM_000059.3	c.5576_5579del	p.Ile1859Lysfs*3	PTC
BRCA2	NM_000059.3	c.5641_5644del	p.Lys1881Glnfs*27	PTC
BRCA2	NM_000059.3	c.5645C>A	p.Ser1882*	PTC
BRCA2	NM_000059.3	c.5904_5907del	p.Val1969Hisfs*34	PTC
BRCA2	NM_000059.3	c.5946del	p.Ser1982Argfs*22	PTC
BRCA2	NM_000059.3	c.5984dup	p.Asn1995Lysfs*8	PTC
BRCA2	NM_000059.3	c.5993_5994del (*n*=2)	p.Gln1998Argfs*4	PTC
BRCA2	NM_000059.3	c.6275_6276del	p.Leu2092Profs*7	PTC
BRCA2	NM_000059.3	c.6629_6630del	p.Glu2210Glyfs*14	PTC
BRCA2	NM_000059.3	c.67+2T>C	p.?	Splicing mutation
BRCA2	NM_000059.3	c.67+3A>G	p.?	Splicing mutation
BRCA2	NM_000059.3	c.7436-2A>T (*n*=2)	p.?	Splicing mutation
BRCA2	NM_000059.3	c.7501C>T	p.Gln2501*	PTC
BRCA2	NM_000059.3	c.7558C>T	p.Arg2520*	PTC
BRCA2	NM_000059.3	c.771_775del	p.Asn257Lysfs*17	PTC
BRCA2	NM_000059.3	c.8167G>C (*n*=2)	p.Asp2723His	Splicing mutation
BRCA2	NM_000059.3	c.994del	p.Ile332Phefs*17	PTC

Abbreviations: LGR, large genomic rearrangement; PTC, premature termination codon.
Recurrence numbers are in brackets.

Table 4 Inactivating mutations detected in other genes than *BRCA1*, *BRCA2* and *TP53*

Gene	*Transcript*	*HGVS nomenclature*	*Expected consequence*	*Mutation type*
ATM	NM_000051.3	c.2250G>A	p.?	Potential splicing mutation
ATM	NM_000051.3	c.2413C>T	p.Arg805*	PTC
ATM	NM_000051.3	c.5496+2T>C	p.?	Splicing mutation
ATM	NM_000051.3	c.5554C>T	p.Gln1852*	PTC
ATM	NM_000051.3	c.790del	p.Tyr264Ilefs*12	PTC
ATM	NM_000051.3	c.8049dup	p.Gln2684Thrfs*4	PTC
BARD1	NM_000465.2	c.176_177del	p.Glu59Alafs*8	PTC
CDH1	NM_004360.3	c.1003_1004insT	p.Arg335Leufs*15	PTC
CHEK2	NM_007194.3	c.1100del (n=2)	p.Thr367Metfs*15	PTC
CHEK2	NM_007194.3	c.1139_1140del	p.Leu380Hisfs*14	PTC
CHEK2	NM_007194.3	c.1461+2T>C	p.?	Splicing mutation
CHEK2	NM_007194.3	c.320-2A>G	p.?	Splicing mutation
CHEK2	NM_007194.3	c.320-5T>A (*n*=2)	p.?	Potential splicing mutation
MLH3	NM_001040108.1	c.3367C>T	p.Gln1123*	PTC
MLH3	NM_001040108.1	c.3466G>A	p.Val1156Ile	Potential splicing mutation
MRE11A	NM_005591.3	c.1096C>T	p.Arg366*	PTC
MRE11A	NM_005591.3	c.1325A>G	p.Lys442Arg	Potential splicing mutation
MRE11A	NM_005591.3	c.504_511del	p.Leu169Argfs*16	PTC
MRE11A	NM_005591.3	c.909_910del	p.Val304Alafs*12	PTC
MSH2	NM_000251.2	c.1147C>T (*n*=2)	p.Arg383*	PTC
MSH2	NM_000251.2	c.1275A>G	p.=	Potential splicing mutation
MSH2	NM_000251.2	c.1386+3A>G	p.?	Potential splicing mutation
MSH2	NM_000251.2	c.2699C>G	p.Ser900*	PTC
NBN	NM_002485.4	c.1142del	p.Pro381Glnfs*23	PTC
NBN	NM_002485.4	c.156_157del	p.Ser53Cysfs*9	PTC
NBN	NM_002485.4	c.37+5G>A (*n*=3)	p.?	Potential splicing mutation
NBN	NM_002485.4	c.38-10T>A	p.?	Potential splicing mutation
NBN	NM_002485.4	c.657_661del	p.Lys219Asnfs*16	PTC
PALB2	NM_024675.3	c.172_175del	p.Gln60Argfs*7	PTC
PALB2	NM_024675.3	c.1972G>T	p.Glu658*	PTC
PALB2	NM_024675.3	c.2325dup (*n*=2)	p.Phe776Ilefs*26	PTC
PALB2	NM_024675.3	c.2386G>T	p.Gly796*	PTC
PALB2	NM_024675.3	c.3362del	p.Gly1121Valfs*3	PTC
PALB2	NM_024675.3	c.696dup	p.Val233Cysfs*2	PTC
PMS1	NM_000534.4	c.2141_2145del	p.Asn714Serfs*2	PTC
PMS2	NM_000535.5	c.1144+2T>G	p.?	Splicing mutation
PMS2	NM_000535.5	c.2523G>A	p.Trp841*	PTC
RAD50	NM_005732.3	c.3036+5G>A	p.?	Potential splicing mutation
RAD50	NM_005732.3	c.3715C>T	p.Arg1239*	PTC
RAD51B	NM_133509.3	c.452+3A>G	p.?	Potential splicing mutation
RAD51C	NM_058216.1	c.622_623del	p.Ile208Leufs*7	PTC
RAD51C	NM_058216.1	c.692C>G	p.Ser231*	PTC
RAD51C	NM_058216.1	c.706-2A>G	p.?	Splicing mutation

carefully considered and the medical implications of a positive test should be clearly explained to the patient before the test. The increased risk of ovarian cancer in MMR mutation carriers led us to include the MMR genes into the NGS panel. In our series, we detected five deleterious MMR mutations in five families whose presentation was not strongly suggestive of Lynch syndrome (Supplementary Figure S3). The low mutation detection rate is counterbalanced by the medical benefit resulting from the identification of a MMR germline mutation.[38,39]

The detection, among 708 patients suspected of HBOC, of 20 inactivating mutations within *PALB2*, *RAD51C*, *CHEK2* and *ATM* (Table 2), indicates that their collective contribution can be estimated at least to 3% and provides another argument highlighting the genetic heterogeneity of HBOC.[19,40] Within families harbouring these mutations, segregation studies will be performed to estimate their causality and penetrance. At the present time, published data concerning the causality of these mutations are still insufficient to integrate these genes into a routine HBOC diagnostic panel. For the other genes of the panel like *MRE11A*, *NBN*, *BARD1* and *BRIP1*, additional studies are needed to validate their implication in HBOC predisposition.

In conclusion, this report shows that the deployment of NGS in medical laboratories significantly increases the throughput, reduces the delay and optimizes the molecular diagnosis of HBOC. Considering the medical consequences of the identification of a deleterious mutation within a family, NGS represents a remarkable progress for

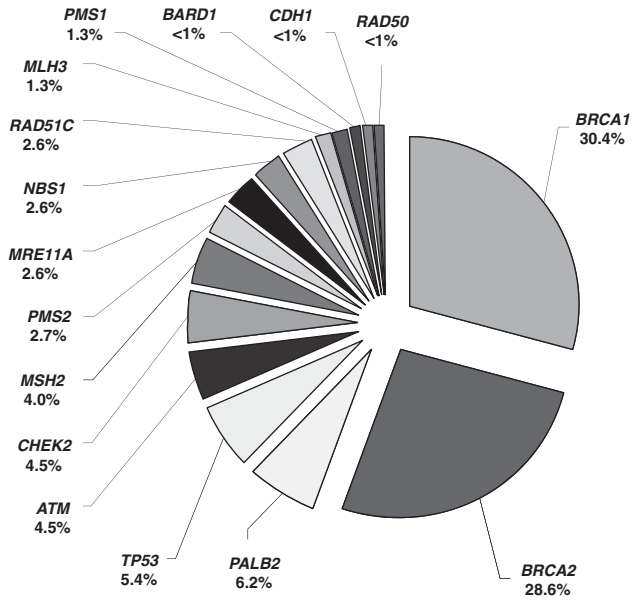

Figure 1 Relative distribution of variants detected with NGS in 708 HBOC patients. Percentages were based on the number of time the gene was sequenced depending on the version of the capture design.

the clinical management of the families. National and international networks of medical laboratories using NGS should facilitate diffusion, evaluation comparison of NGS tools and software. The second challenge for laboratories performing HBOC diagnosis will be the interpretation of mutations identified, in particular, within the other genes than *BRCA1* and *BRCA2*. Even more than in the pre-NGS era, the creation of databases of clinical grade and the interaction with clinicians will be essential.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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