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ARTICLE

Post-mortem testing; germline *BRCA1/2* variant detection using archival FFPE non-tumor tissue. A new paradigm in genetic counseling

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Accurate estimation of cancer risk in HBOC families often requires *BRCA1/2* testing, but this may be impossible in deceased family members. Previous, testing archival formalin-fixed, paraffin-embedded (FFPE) tissue for germline *BRCA1/2* variants was unsuccessful, except for the Jewish founder mutations. A high-throughput method to systematically test for variants in all coding regions of *BRCA1/2* in archival FFPE samples of non-tumor tissue is described, using HaloPlex target enrichment and next-generation sequencing. In a validation study, correct identification of variants or wild-type was possible in 25 out of 30 (83%) FFPE samples (age range 1–14 years), with a known variant status in *BRCA1/2*. No false positive was found. Unsuccessful identification was due to highly degraded DNA or presence of large intragenic deletions. In clinical use, a total of 201 FFPE samples (aged 0–43 years) were processed. Thirty-six samples were rejected because of highly degraded DNA or failed library preparation. Fifteen samples were investigated to search for a known variant. In the remaining 150 samples (aged 0–38 years), three variants known to affect function and one variant likely to affect function in *BRCA1*, six variants known to affect function and one variant likely to affect function in *BRCA2*, as well as four variants of unknown significance (VUS) in *BRCA1* and three VUS in *BRCA2* were discovered. It is now possible to test for germline *BRCA1/2* variants in deceased persons, using archival FFPE samples from non-tumor tissue. Accurate genetic counseling is achievable in families where variant testing would otherwise be impossible.

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INTRODUCTION

There has been a tremendous technological development, since the discovery and cloning of the *BRCA1* (breast cancer 1, early onset) gene in 1994[1] and shortly after the *BRCA2* (breast cancer 2, early onset) gene in 1995.[2] Today, testing for variants in the two genes is a widespread option, when counseling families with high risk of breast and ovarian cancer (HBOC). Women harboring a germline variant known to affect function in *BRCA1* or *BRCA2* are confronted with a lifetime risk of breast cancer of 60–80% and a risk of ovarian cancer of 20–50%[3,4] as well as risk of other cancers like pancreatic cancer and malignant melanoma.[5] Men harboring the same variants are facing an increased risk of prostate cancer and breast cancer.[5] Many variant carriers will choose prophylactic surgery to reduce their cancer risk or may enter more extensive screening programs, to detect cancer in an early stage to improve the outcome.[6]

Until now, testing for *BRCA1* and *BRCA2* variants (or germline variants in other high-risk genes), using traditional methods, required a blood sample, saliva sample or buccal smear from a living person or archived fresh frozen tissue or blood from a deceased person, in order to obtain high-quality DNA for the analysis. This has ruled out families, in which the relatives suffering from breast or ovarian cancer have already died. In such families, where, eg, a young woman is seeking genetic counseling, and her mother or other close relatives died from breast or ovarian cancer at a young age (eg, a decade ago), there will be no options for variant testing. If a variant testing is carried out in the woman seeking genetic counseling, a negative result will be difficult to interpret and cannot be used to predict her risk of breast and ovarian cancer. Today, the only option for such families is to use 'indirect' testing, where variant testing is offered to close relatives (siblings and children to the deceased person) to search for a germline variant.[7] It is recommended to test at least 3–4 first-degree relatives, in order to increase the probability to identify or exclude a variant, which can make indirect testing costly and laborious. Furthermore, it can sometimes be difficult (or impossible) to get blood samples from relatives. Lastly, in many countries, indirect testing will not be covered by health insurance.

Previously, several attempts have been made to test for variants in archival formalin-fixed, paraffin-embedded (FFPE) tissue,[8,9] but until now, only the Ashkenazi Jewish founder mutations have successfully been tested for in FFPE samples.[10,11] However, outside the Ashkenazi Jewish community, testing for specific (founder) variants is insufficient for accurate risk assessment and counseling of families with increased risk of breast and ovarian cancer, and unknown *BRCA* status.

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A new routine, high-throughput analysis to test archival FFPE samples of non-tumor tissue for germline variants in *BRCA1* and *BRCA2* was introduced using HaloPlex target enrichment (Agilent, Midlothian, Scotland/UK) and next-generation sequencing technology (Illumina, San Diego, CA, USA), to determine whether a deceased relative harbored a germline *BRCA1* or *BRCA2* variant known to affect protein function. The results of the initial validation study, including 32 samples, and the first clinical experience, including 201 samples from deceased relatives, with this new FFPE testing analysis are described here in detail.

MATERIALS AND METHODS

FFPE tissue samples

In the validation study, 32 FFPE samples of non-malignant tissue, from women with a known *BRCA1* or *BRCA2* variant or wild type (women previously tested for *BRCA* variants) were chosen from families known at the Department of Clinical Genetics, Vejle hospital. The samples were chosen to include a wide range of variant types (frameshift, missense, small indels, splice site and large deletions) and the age of the tissue ranged from 1 to 14 years. Tissue samples were included based on availability sufficient amount of non-malignant tissue (no small biopsies) and tissue containing a substantial amount of nuclei (eg, not fat tissue). The FFPE samples were investigated by an experienced pathologist and 9×15 μm FFPE tissue sections were cut. If a sufficient amount of DNA was not gained from 9×15 μm sections, further 9×15 μm sections were cut and DNA was extracted. A maximum of 18×15 μm sections were used in three samples (Val6, Val19 and Val22).

Furthermore, all information regarding tissue type, age of tissue and variant status was blinded to the technical and bioinformatic staff, and blinding was only lifted after disclosure of the final report of *BRCA1/2* variants in the validation study.

In the clinical study, we used the best available tissue, evaluated by an experienced pathologist. If optimal tissue was not available, less optimal tissue was used (in four cases, tissue containing malignant cells were used). In some samples more than 9×15 μm sections were used to obtain sufficient amount of DNA for the analysis.

DNA extraction

DNA was extracted from three tubes each containing 3×15 μm FFPE tissue sections per sample using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to *QIAamp DNA FFPE Tissue Handbook* with a few modifications (www.qiagen.com). The DNA was validated using 1% Tris-acetate EDTA gel electrophoresis and DNA concentrations were determined using PicoGreen (Invitrogen, Life Technologies Europe BV, Nærum, Denmark). To verify overall quality of the DNA extracted from FFPE samples, the level of fragmentation was estimated using a quality control assay (QC assay) based on PCR provided by Agilent (Agilent Technologies, 2012). HapMap DNA (NA12878) sample was used as a non-degraded control (Coriell Institute). According to the results of the QC assay, samples were classified as good (26), medium (2) or poor (2), see Table 1. Furthermore, all DNA samples were analyzed on an Agilent TapeStation (Agilent Technologies) using Genomic ScreenTape and reagents according to Agilent gDNA ScreenTape System Quick Guide. To compensate for a higher level of fragmentation, the amount of input DNA for further analysis was 225, 500 and 1000 ng for the samples classified as good, medium or poor, respectively. A flowchart of the DNA quality assessment before NGS library preparation is shown in Figure 1.

HaloPlex target enrichment and sequencing

Twenty-nine genes encoding *BRCA1/2* and other important proteins involved in the homologous recombination pathway,[12,13] were included in the HaloPlex (Illumina 100) custom design of 552 targets with a 118889-bp region of interest (ROI) (Agilent Technologies, 2012).[14] The fraction of bases in ROI that can be analyzed covered 98.8% of the target region. HaloPlex libraries were constructed according to manufacturer's protocol v.D4 (Agilent Technologies, 2013).[15] Indexes were incorporated for each sample during enrichment, allowing samples to be multiplexed before sequencing. A total of 30 HaloPlex libraries were validated on a bioanalyzer High Sensitivity chip (Agilent Technologies).

After enrichment, HaloPlex libraries were diluted to 10 nM, pooled, denatured and subjected to paired-end (2×150 bp), single index (8 bp) reversible terminator based DNA sequencing on a MiSeq (Illumina).

In both parts of the study only data regarding *BRCA1* and *BRCA2* were analyzed.

Alignment

For each sequenced sample, the raw fastq files generated from the Illumina MiSeq system were trimmed with TrimGalore (version 0.33), subsequently mapped to the hg19 human reference genome using MOSAIK (version 2.2),[16] and converted to BAM using Samtools (version 0.1.19).[17] Each sample BAM file was preprocessed with Genome Analysis Toolkit [18,19] (GATK version 3.1.1; local realignment around indels and base quality score recalibration), before variant calling. General alignment statistics (eg, number of aligned reads, size of insert fragment, etc) were generated with BAMtools (version 2.3.0).[20] Target-specific alignment statistics (ie, per base-/region-/gene-/sample-coverage and coverage percentage of ROIs), were obtained using GATK DepthOfCoverage.

Variant calling and annotation

Following preprocessing of BAM files, variant calling was performed using GATK HaplotypeCaller (GATK version 3.1.1). Low-quality/false positive variants were filtered out using GATK VariantFiltration. Only variants fulfilling the following criteria FS$<$250 & QD$>$2.0 & QUAL$>$200 & Homopolymer-Run (HRun)$<$7 & DP$>$10, were kept in the filtered single-sample variant call sets, which were subsequently merged to produce a multi-sample call set for all validation samples or clinical samples, respectively. Each merged call set was annotated using SnpEff (version 3.6)[21] and VariantTools (version 2.3),[22] using build-in and custom annotation tracks. NCBI reference sequences (RefSeq) NM_007294.3 and NM_000059.3 have been used for the annotation of *BRCA1* and *BRCA2* variants, respectively. These RefSeq transcripts are included in the Locus Reference Genomic (LRG) data LRG_292-BRCA1 and LRG_293-BRCA2. BRCA1/2 variant data has been submitted to Leiden Open Source Database at http://databases.lovd.nl/shared/individuals/PatientID (PatientID: 00051505–00051521). Analysis of coverage data and quality metrics were performed in R (version 3.0.2 Frisbee sailing),[23] using base packages and the CRAN package 'pheatmap' for heatmap representation of coverage data.[24] Heatmap clustering is based on complete linkage on Euclidian distances. Spearman's Rho was used to assess the correlation between FFPE age and ROI coverage.

Deletion/duplication testing using MLPA

Clinical FFPE DNA samples were subjected to mulitiplex ligation-dependent probe amplification (MLPA). A total 5 μl of FFPE DNA was used for each MLPA reaction, and analysis was conducted according to manufactures one-tube protocol (MRC-Holland, Amsterdam, The Netherlands). SALSA MLPA P002 *BRCA1* probemix and SALSA MLPA P045 *BRCA2*/*CHEK2* probemix were used for the MLPA analysis of *BRCA1* and *BRCA2*, respectively. Fragment separation was conducted on an ABI3130 using POP6 polymer and 36 cm capillaries. Injection mixture contained 0.5 μl MLPA PCR reaction and 12 μl Hi-Di formamide master mix (0.5 μl GS-500 MW marker+12 μl Hi-Di formamide). Run module: FragmentAnalysis; injection voltage: 1.4 kV; injection time 15 s; run voltage: 15 kV; run time: 2400 s; and oven temperature: 55 °C. The fragment analysis results were analyzed using GeneMapper and GeneMarker. The RefSeq transcripts NM_007294.3 and NM_000059.3 are included in the LRG data LRG_292-BRCA1 and LRG_293-BRCA2, respectively. The LRG-specific exon numbering for *BRCA1* and *BRCA2* has been used.

Ethical considerations

Since the validation study only involved a new method for finding known variants and no new knowledge about the participant's genetic status was gained, we were allowed to perform the genetic investigations without prior consent from the subjects who participated with their tissue samples. This permission was granted by the Regional Committee on Health Research Ethics, Region of Southern Denmark. In the clinical setting, all investigations on tissue

Table 1 Validation study results

Sample	FFPE age	Tissue type	QC	BRCA	HGVS	Designation BIC	Median fragment length (bp)	Mapped reads	30x Cov All (%)	30x Cov BRCA1 (%)	30x Cov BRCA2 (%)	Positive match
Val1[a]	2	Breast	Good	1	c.(80+1_81−1)_(4986+1_4987−1)del	—	113	4 013 545	96.6	91.1	96.0	No
Val2	1	Ovary	Good	—	—	—	110	983 590	91.2	86.6	91.4	wt
Val3	4	Ovary	Good	2	c.6373_6373delA	6601delA	113	957 896	90.9	88.8	89.3	Yes
Val4	6	Breast/papilla	Good	1	c.2475_2475delC	2594delC	116	1 142 962	95.0	95.4	93.8	Yes
Val5[a]	7	Cervix	Medium	2	c.145G>T	E49X	103	2 029 254	88.0	85.4	85.6	Yes
Val6[a]	12	Cervix	Good	1	c.2475_2475delC	2594delC	94	1 062 269	70.8	75.6	66.2	Yes
Val7	5	Cervix	Good	2	c.6373_6373delA	6601delA	114	1 100 509	93.8	93.3	92.7	Yes
Val8	5	Ovary	Good	2	c.145G>T	E49X	121	2 078 508	97.5	99.1	97.4	Yes
Val9[a]	11	Cervix	Good	—	—	—	89	1 787 300	76.0	74.9	70.6	wt
Val10	2	Breast	Good	1	c.5467+1G>A	IVS23+1G>A	117	2 952 445	97.3	98.9	97.1	Yes
Val12[a]	10	Ovary	Good	—	—	—	103	3 009 303	89.1	86.3	86.2	wt
Val13	2	Ovary	Good	1	c.3710_3710delT	3829delT	154	977 476	97.8	99.6	97.7	Yes
Val14[a]	9	Cervix	Good	1	c.(80+1_81−1)_(4986+1_4987−1)del	—	110	4 242 148	94.6	90.5	92.9	No
Val15[a]	12	Uterus	Medium	2	c.9106C>T	Q3036X	90	1 431 801	74.9	77.2	69.4	No
Val16[a]	13	Appendix	Good	—	—	—	84	1 831 019	70.6	68.3	65.3	wt
Val18[a]	10	Ovary	Good	1	c.2475_2475delC	2594delC	106	3 642 186	93.1	92.3	92.7	Yes
Val19[a]	11	Breast/fat	Good	1	c.5467+1G>A	IVS23+1G>A	101	1 164 860	77.1	80.3	72.4	Yes
Val20	3	Lymph node	Good	1	c.2475_2475delC	2594delC	102	1 805 689	88.7	84.4	88.2	Yes
Val21	8	Lymph node	Good	2	c.145G>T	E49X	110	1 474 722	92.4	88.8	91.5	Yes
Val22[a]	6	Breast/fat	Good	2	c.6373_6373delA	6601delA	113	4 511 857	95.5	95.8	93.2	Yes
Val23	6	Breast	Good	2	c.7617+1G>A	IVS15+1G>A	130	7 449 873	99.3	100.0	99.7	Yes
Val24[a]	13	Skin/fat	Good	1	c.2475_2475delC	2594delC	100	2 628 280	84.9	78.8	78.5	Yes
Val25	5	Breast	Good	2	c.6373_6373delA	6601delA	115	1 075 069	95.0	95.4	94.5	Yes
Val26[a]	14	Uterine	Poor	1	c.(80+1_81-1)_(4986+1_4987-1)del	—	114	1 911 149	90.1	88.6	84.6	No
Val27	6	Ovary	Good	1	c.3319G>T	E1107X	114	745 661	90.9	86.0	90.2	Yes
Val28[a]	12	Lymph node	Good	2	c.6373_6373delA	6601delA	105	2 636 555	88.8	81.1	86.8	Yes
Val29[a]	6	Breast	Poor	1	c.5263_5264insC	5382insC	69	1 411 592	48.7	47.7	41.4	No
Val30	4	Gall bladder	Good	—	—	—	118	1 532 684	96.9	96.7	97.0	wt
Val31	4	Breast	Good	1	c.3710_3710delT	3829delT	114	6 451 329	97.4	96.3	96.9	Yes
Val32	4	Ovary	Good	2	c.7617+1G>A	IVS15+1G>A	114	939 122	92.2	89.0	91.3	Yes

Abbreviations: BIC, Breast Cancer Information Core; bp, base pairs; FFPE, formalin-fixed, paraffin-embedded; HGVS, human Genome Variation Society; QC, quality control; wt, wild type.
[a]15 FFPE HaloPlex enriched libraries were re-sequenced due to low BRCA1/2 target coverage. Merged sequencing data from the two sequencing runs are shown in this table.

samples from deceased persons, was performed only after informed consent from a closely related family member seeking genetic counseling, according to standard practice in clinical genetics.

RESULTS

Correct call of pathogenic variants in BRCA1 and BRCA2

DNA extraction was successful in 30 out of 32 FFPE samples from women with a known *BRCA1/2* variant or wild-type. In two samples, the amount of DNA was too low (sample 11 and 17) to perform target enrichment, library preparation and sequencing. In 25 out of 30 sequenced samples of non-cancer FFPE tissue, it was possible to correctly identify and classify either a *BRCA1* or *BRCA2* variant (true positive: 20 samples) or wild type (true negative: five samples), resulting in an accuracy of 83.3%, see Table 1. In three samples (Val1, Val14 and Val26), it was not possible to identify a large intragenic deletion c.(80+1_81−1)_(4986+1_4987−1)del corresponding to the deletion of exons 3–15 in *BRCA1*. Furthermore, two samples did not result in a correct variant call, due to poor DNA quality, and hence a low coverage (9x) at the position of interest (Val29) and a skewed read distribution with 81% and 19% of read data supporting the reference and variant allele, respectively (Val15). Consequently, a false negative result was observed in a total of 5 of the 30 samples. However, the variant of Val15 was correctly called in the raw data, but was filtered out as a result of the skewed read distribution. No additional (ie, false positive) variants affecting protein function were identified (false discovery rate: 0.0). Albeit pertaining to a limited number of samples, these findings indicate that the method and analysis strategy used provides high sensitivity (0.8) and very-high specificity (1.0) and positive prediction value (1.0). In contrast, the five false negative calls and the inclusion of only five true negative samples results in a lower negative prediction value (0.5).

MLPA was not applied in the validation study.

Target performance: validation study

In this study, the ROI is defined as coding exons plus 20-bp flanking region. The ROI coverage was not uniform across the samples as 30× coverage varied between 48.7 and 99.3% for all 29 target genes (All), 47.7–100% for *BRCA1* and 41.4–99.7% for *BRCA2*, see Table 1. This is also illustrated in the heatmap, see Figure 2a, where an overall good coverage was found in 24 out of the 30 samples. In six samples, the

coverage was more diverse across the 29 target genes; Val29 had the lowest 30x coverage percentage across all target genes, indicated by the blue color in the heatmap. Besides Val29, five samples (Val6, Val9, Val15, Val16 and Val19) had a low-medium (<65%) 30x coverage percentage across seven out of the 29 target genes. Common for these five samples was that the FFPE sample age was more than 10 years upon DNA purification. The age of FFPE samples varied from 1 to 14 years. A significant inverse correlation between the age of FFPE sample (years) and the percentage of ROIs with at least 30x coverage ($\rho = -0.598$, $P < 0.01$) was detected, see Figure 2b. Nevertheless, some samples aged 10 or more years (Val12, Val18, Val24, Val26 and Val28) still resulted in a high percentage of ROIs (>80%) with at least 30x coverage. Furthermore, a strong correlation between the median fragment length (bp) sequenced and the percentage of ROIs with at least 30x coverage ($\rho^2 = 0.914$, $P < 0.001$) was detected as well.

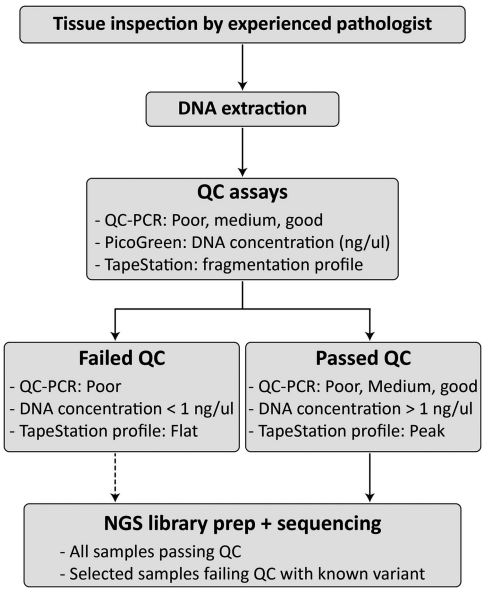

Figure 1 Flowchart of FFPE DNA sample and QC assays. After DNA extraction, three QC assays were performed to validate the quality of the DNA: (1) QC-PCR was used to estimate the level of fragmentation by comparing two PCR products amplified from FFPE DNA with the amplified PCR products from HapMap DNA (NA12878). According to the results of the QC-PCR, samples were classified as good, medium or poor. (2) DNA concentrations were measured using a PicoGreen assay. (3) All DNA samples were analyzed on a TapeStation to view the fragmentation profile of the DNA. Either the profiled was rated as 'flat' indicating that DNA was highly degraded or not present, or the profile was rated as 'peak' indicating that the DNA was degrade but had a peak when looking at the electropherogram. If a sample was rated poor, had a DNA concentration less than 1 ng/μl and a 'flat' fragmentation profile, the DNA sample had failed QC. Only selected DNA samples failing QC were passed on to library preparation and sequencing, if there was a known variant in the family to search for.

Target performance: Clinical Samples

In the clinical data, the ROI coverage was not uniform across the samples as 30x coverage varied between 21.1 and 99.5% for all 29 target genes, 18.1 and 99.8% for *BRCA1* and 15.1 and 99% for *BRCA2*. As seen in the validation study, a significant inverse correlation between age of FFPE sample (years) and the percentage of ROIs with at least 30x coverage $\rho = -0.386$ $P < 0.01$) was detected. Even though some samples performed inadequate regarding ROI coverage, positive results were obtained in some of these samples, see Figure 3. As an example, a variant known to affect protein function in *BRCA1* was found in sample D13-2662, even though the percentage of ROI with at least 30x coverage (all) was low (24.8%), see Table 2.

Clinical experience with 201 samples from deceased persons

DNA was successfully extracted from 201 clinical FFPE samples from deceased relatives from families with a high suspicion of carrying a *BRCA1* or *BRCA2* variant, based on clinical experience or using the BOADICEA risk estimation program.[25] The age of the samples ranged from 0 to 43 years. Based on the results of the QC assay, 23 samples were rejected for further analyses. The remaining 178 FFPE DNA samples were subjected to target enrichment library preparation. 13 samples were removed due to failed library preparation, and the remaining 165 FFPE samples, aged 0–38 years, were successfully sequenced and subjected to BRCA data analysis, see Figure 4.

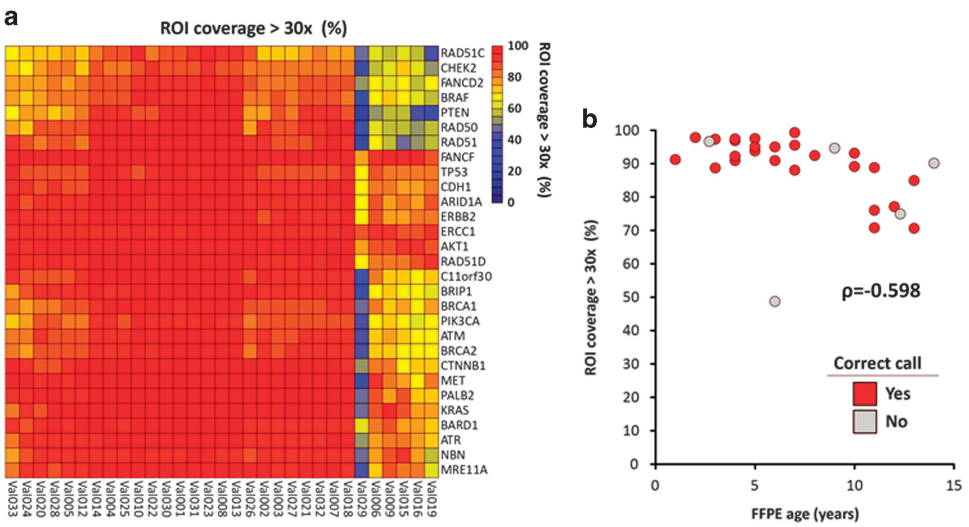

Figure 2 (**a**) Heatmap of all 30 sequenced samples from the validation study. Red color represents that 100% of ROI is covered at least 30x times whereas blue color represents 0% coverage. Each column represents one sample and each row the gene sequenced. (**b**) Validation study: an inverse correlation between age of the sequenced archival FFPE sample and the percentage of 30x coverage of ROI, ($\rho^2 = -0.598$, $P < 0.01$). Red dots represent correct call of variant status, gray dots represent incorrect calls.

After BRCA data analysis, 15 out of the 165 samples were analyzed primarily to search for a known familial *BRCA1/2* variant, by visual inspection of the known genomic position.

In the 150 FFPE samples, a total of three variants known to affect function, and one variant likely to affect function in *BRCA1*, six variants known to affect function, and one variant likely to affect function in *BRCA2*, four VUS in *BRCA1* and three VUS in *BRCA2* were detected (Table 2 shows all the described variants in detail). In the remaining 133 samples, no variants or only benign/likely benign variants were found. In the 15 samples analyzed because of a known familial variant (or VUS), seven variants known to affect function in *BRCA1* and two variants known to affect function in *BRCA2*, as well as one VUS in *BRCA1* and three VUS in *BRCA2* were found (Table 3 shows all the described variants in detail).

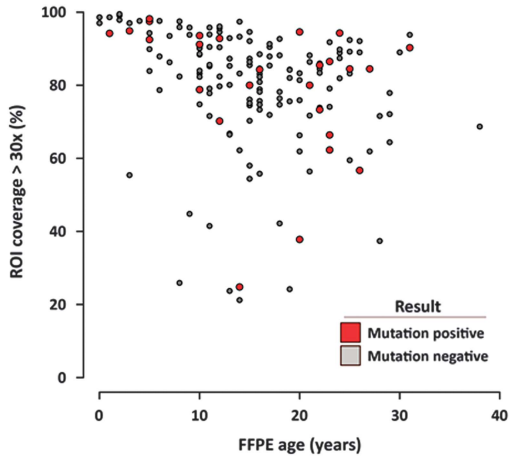

Figure 3 Clinical FFPE Samples: correlation between age of the 165 sequenced archival FFPE samples and 30x coverage of ROI, ($\rho = -0.0386$, $P < 0.01$). Red dots represent a positive finding of a variant known to affect function or VUS in *BRCA1/2*, gray dots represent negative findings (no variants).

In three samples more than one variant was found; in sample D14-1242, two VUS in *BRCA1* c.1486C>T and c.5297T>A (HGVS) were identified, see Table 2. In sample D14-1243, a biopsy from the gastric mucosa harboring an adenocarcinoma, two variants in *BRCA1* were found, see Table 3. The first, a familial variant known to affect *BRCA1* function; c.427G>T (HGVS) was correctly identified and a second variant c.4043_4043delG (HGVS) was also detected, and is assumed to be of somatic origin. The second variant is not known in the Breast Cancer Information Core database, but it induces a frameshift leading to a premature stop codon. In sample D14-1837, a *BRCA1* variant known to affect function c.427G>T was found together with a VUS in *BRCA2* c.6287C>T, see Table 3.

MLPA analysis was used to detect larger intragenic deletions or duplications. The *BRCA1* and *BRCA2* MLPA results were normal in 90 and 80 samples, respectively, but non-informative because of low DNA quality in 87 and 97 samples, respectively. *BRCA1* and *BRCA2* MLPA analysis were not performed in one sample. No large deletions or duplications in *BRCA1/2* were found in the clinical samples.

DISCUSSION

To our knowledge, this is the first published successful attempt to systematically test archival FFPE samples of non-cancer tissue for

Table 2 BRCA1/2 Variants known to affect function and variants of unknown significance found in clinical samples

Sample	FFPE age	Tissue type	QC	BRCA	HGVS	BIC	Category Class IARC	Aligned Reads	30x Cov All (%)	30x Cov BRCA1 (%)	30x Cov BRCA2 (%)	Median fragment length (bp)
D11-2183	13	Uterus	Poor	2	c.2830A>T	K944X	5	4 390 169	78.8	79.5	75.5	97
D11-2285	22	Uterus	Medium	1	c.5213G>A	G1738E	4	749 583	73.3	73.1	67.5	103
D12-1078	20	Fatty	Poor	2	c.1526G>T	NA	VUS	178 778	37.8	33.6	29.3	112
D12-1671[a]	12	Areola	Good	2	c.4258_4258delG	4486delG	5	633 324	78.8	77.2	75.2	111
D13-2657	17	Ovary[b]	Poor	2	c.7480C>T	R2494X	5	4 301 047	84.3	80.8	79.6	104
D13-2660[c]	2	Fallopian tube	Medium	1	c.427G>T	E143X	5	4 569 459	94.2	95.0	94.0	114
D13-2662	14	Lymph node	Poor	1	c.5559C>A	NA	5	190 112	24.8	18.1	16.7	69
D13-2877	6	Ovary	Medium	2	c.6943A>C	I2315L	VUS	1 038 886	92.5	91.5	91.3	113
D14-1242	5	Gall bladder	Good	1	c.1486C>T	R496C	VUS	2 908 225	97.5	91.9	96.7	86
D14-1242				1	c.5297T>A	I1766S	VUS					
D14-1779	20	Glandula submandibularis	Good	1	c.4862A>G	NA	VUS	2 722 418	94.6	89.9	93.2	80
D14-1912	26	Lymph node	Poor	2	c.7878G>C	W2626C	4	411 696	56.7	49.2	51.5	61
D14-2457[d]	5	Gall bladder	Medium	2	c.5645C>A	S1882X	5	2 290 163	98.2	95.7	96.5	61
D14-2458[c]	24	Cervix	Medium	1	c.3008_3009delTT	3127delTT	5	2 636 934	94.3	89.1	92.4	80
D14-3407	25	Uterus	Medium	2	c.5966C>A	NA	5	1 153 260	84.5	85.0	78.2	70
D14-3408	15	Lymph node[e]	Medium	2	c.6082_6086delGAAGA	6310del5	5	1 181 269	80.0	75.1	73.2	69
D15-0122	12	Lymph node	Good	2	c.9187C>T	P3063S	VUS	3 681 704	92.8	88.1	91.3	71
D15-0361	10	NA	Good	1	c.1486C>T	R496C	VUS	1 969 218	91.2	88.7	89.8	76

Abbreviations: BIC, Breast Cancer Information Core; bp, base pairs; FFPE, formalin-fixed, paraffin-embedded; HGVS, Human Genome Variation Society; IARC, International Agency for Cancer Research; NA, not available; QC, quality control; VUS, variant of unknown significance.
The BRCA1/2 variants are classified according to the proposed classification system for sequence variants identified by genetic testing by Plon and coworkers for the IARC Unclassified Genetic Variants Working Group 2008. Only class 5 (definitely pathogenic), class 4 (likely pathogenic) and class 3 (VUS) are reported in this table. BRCA1/2 variant data have been submitted to Leiden Open Source Database at http://databases.lovd.nl/shared/individuals/PatientID (PatientID: 00051505–00051521).
[a]Detected in a living relative afterwards.
[b]Ovary tumor. Tumor content is estimated to 20%.
[c]Detected in another FFPE sample from a relative.
[d]Verified with Sanger DNA sequencing from a second FFPE patient sample.
[e]Lymph node. Tumor content is estimated to 50%.

germline variants in *BRCA1/2*. Previous attempts, using more 'classical' methods, such as single-strand conformation polymorphism analysis or sanger sequencing, resulted in a substantial rate of both false positive and false negative results[8,9] or at best was limited to search for the known Jewish Ashkenazi founder mutations.[10,11,26] However, a reliable NGS method for detection of variants in *BRCA1/2* in FFPE samples from tumor tissue was recently published.[27]

In the validation study, the variant calling resulted in a true match in 25 out of 30 sequenced samples (83%). It was not possible to detect a large intragenic deletion in *BRCA1* in three samples (Val1, Val14 and Val26). However, this was expected because of the choice of sample preparation method, which is based on an amplicon target-enrichment technique with non-random DNA shearing, making copy number variation detection difficult, since duplicate reads cannot be identified. Based on this, it is recommended to use MLPA or a similar method for the detection of larger deletions or duplications, although analyzing highly degraded DNA may cause inconclusive results. Furthermore, it was not possible to correctly call a single base substitution (Val15) or a single base insertion (Val29) in two samples. However, after unblinding of the study, the variant of Val15 could be correctly called in the raw data, but was filtered out as a result of the skewed read distribution between wild type and alternative. By changing the settings in the data analysis pipeline, it was possible to correctly call this variant. In the case of Val29, the coverage was low (9x coverage), and the variant was not detected in any reads covering this position. Another important result is that we did not find any false positive variants, in either *BRCA1/2*-positive or in the wild-type group. Formalin fixation is known to introduce alterations in the DNA and this may result in false positive findings, which has been reported from earlier attempts.[9]

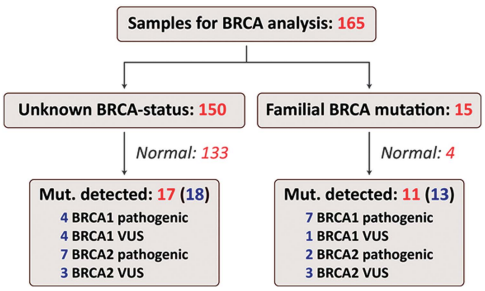

Figure 4 BRCA data analysis of 165 clinical FFPE samples: a total of 18 variants were detected in 17 out of the 150 FFPE samples with unknown BRCA-status. A total of three variants known to affect function and one variant likely to affect function in *BRCA1*, six variants known to affect function and one variant likely to affect function in *BRCA2*, four VUS in *BRCA1* and three VUS in *BRCA2* were detected. In the 15 samples analyzed because of a familial variant known to affect function in *BRCA1/2* (or VUS), 13 variants were detected in 11 samples. A total of seven variants known to affect function in *BRCA1* and two variants known to affect function in *BRCA2*, as well as one VUS in *BRCA1* and three VUS in *BRCA2* were detected. Number of samples are written in red, and number of variants are written in blue.

The DNA quality is a strong predictor of the outcome of the analysis, and a significant correlation between the median fragment length (bp) of the sequenced DNA and 30x coverage percentage across the ROI supports this observation. Highly degraded DNA contains shorter DNA fragments and therefore more DNA is required to obtain successful target enrichment. However, based on this study we recommend using $9 \times 15\,\mu$m sections for DNA extraction. As seen from Figure 2, coverage is decreasing with age of the tissue (especially after 10 years of age), implying that DNA quality decreases with age, but with large variation, as some samples aged 10 or more years may still result in a high 30x coverage.

When implementing the test in our clinical setting, a greater variation in both age of the tissue, coverage and hence outcome of the sequencing was detected compared to the validation study. The age of the clinical samples was up to 43 years, but DNA extracted from

Table 3 Familial BRCA1/2 testing in FFPE samples and verification of variants detected during BRCA1/2 FFPE testing

Sample	FFPE age	Tissue type	QC	BRCA	HGVS	BIC	Category Class	Aligned Reads	30x Cov All (%)	30x Cov BRCA1 (%)	30x Cov BRCA2 (%)	Median fragment length (bp)
D14-1243[a]	3	Biopsy[b]	Good	1	c.427G>T	E143X	5	1 718 979	94.9	89.2	93.8	87
D14-1243[c]				1	c.4043_4043delG	NA	5					
D14-1397[d]	10	Endometrium	Medium	2	c.6455C>A	S2152Y	VUS	2 155 856	93.6	89.1	92.0	81
D14-1662[d]	31	Endometrium	Medium	1	c.3710_3710delT	3829delT	5	2 908 904	90.3	87.9	86.6	70
D14-1837[d]	22	Breast	Medium	1	c.427G>T	E143X	5	1 912 039	85.5	84.0	83.7	68
D14-1837	22	Breast	Medium	2	c.6287C>G	NA	VUS	1 912 039	85.5	84.0	83.7	68
D14-1879[a]	23	Ovary	Medium	1	c.3008_3009delTT	3127delTT	5	1 308 529	86.5	86.4	82.5	72
D14-2457[d]	5	Gall Bladder	Medium	2	c.5645C>A	S1882X	5	2 290 163	98.2	95.7	96.5	61
D14-2518[d]	12	Thorax	Medium	1	c.3477_3479delAAAinsC	3596delAAAinsC	5	505 127	70.2	65.4	67.5	69
D14-2551[d]	27	Endometrium	Medium	1	c.4096+3A>G	IVS11+3A>G	VUS	1 044 811	84.5	81.3	80.7	70
D14-3279[d]	23	Cervix	Medium	1	c.3710_3710delT	3829delT	5	330 837	62.3	58.3	61.1	68
D14-3281[d]	23	Cervix	Medium	2	c.1813_1813delA	2041delA	5	314 287	66.4	59.0	62.1	77
D15-0246[d]	21	Lymph node[e]	Medium	2	c.9501+3A>T	IVS25+3A>T	VUS	1 970 700	80.0	72.7	76.1	66

Abbreviations: BIC, Breast Cancer Information Core; bp, base pairs; FFPE, formalin-fixed, paraffin-embedded; HGVS, Human Genome Variation Society; IARC, International Agency for Cancer Research; NA, not available; QC, quality control; VUS, variant of unknown significance.
The BRCA1/2 variants are classified according to the proposed classification system for sequence variants identified by genetic testing by Plon and coworkers for the IARC Unclassified Genetic Variants Working Group 2008. Only class 5 (definitely pathogenic), class 4 (likely pathogenic) and class 3 (VUS) are reported in this table.
[a]Verification of detected variant found in a FFPE sample from a relative.
[b]Biopsy of gastric mucosa adenocarcinoma.
[c]The second BRCA1 pathogenic mutation found in D14-1243 could be a somatic mutation.
[d]Known familial variant detected in a living relative.
[e]Lymph node. Tumor content is estimated to 40%.

tissue older than 38 years did not meet our quality criteria for library preparation and sequencing. The percentage of 30x coverage of the target genes varied more compared to the validation data set. Even though, the coverage is declining with increasing age, variants were detected in samples with coverage in the lower range, see Figure 3. These results represent the cohort tested with the current analytical set-up, and since no true information exists regarding incidence of *BRCA1/2* mutations in this cohort, it is impossible to calculate neither sensitivity nor specificity of the test. The overall negative result from the MLPA analysis comes as no surprise, as deletions in *BRCA1/2* are found in a minority (3.8%) of the *BRCA1/2*-positive families in Denmark.[28]

Detecting a variant known to affect function in *BRCA1/2* in a FFPE sample should always prompt further investigation. A variant should always be verified in a new sample, either from a different tissue from the same person, or should be verified in another family member. Before a variant is verified, it can only be assumed positive, since there is a risk of false positive findings due to false positive calls from software, PCR amplification, sequencing error, or due to alterations in the DNA caused by the fixation or age.[29] Negative results (normal sequence) should always be interpreted with caution. If the 30x coverage is high (we recommend $>90\%$), the result may be interpreted, as if it was a blood sample from the same person. However, the possibility of a variant in the ROI that is not covered can never be excluded. If coverage is low, risk estimation should be offered, as if no analysis was performed and based solely on the family history. In families where a VUS is found, FFPE testing could potentially be used to perform segregation analysis to analyze, whether the variant co-segregate with the disease in a family. FFPE testing could also be used to investigate if a variant was inherited from the maternal or paternal side of the pedigree. This could potentially reduce anxiety and the economic burden of testing family members on both sides of a family, instead of testing only the relevant side, after identifying the variant in either the mother or father (if a tissue sample is available).

Testing FFPE samples may be used for other purposes than *BRCA1/2* testing. As the percentage of 30x coverage across the 29 target genes in the panel used, is generally consistent, the method can be used to test for variants in other highly penetrant breast and ovarian cancer genes (like *PALB2, RAD51C/D, PTEN, CDH1* and *TP53*). The usefulness of the test is anticipated to increase with mortality risk of the investigated gene. If there is a high mortality rate in carriers of, eg, *TP53* or *CDH1* variants, it will be less likely that there is a living carrier to investigate. We used normal tissue, in order to search for germline variants, but the method could also be applied to malignant tissue, in order to search for somatic variants in genes involved in the homologous recombination pathway (the BRCAness genes).[30] Finding somatic variants in one of these genes could be important for future management of cancer with a potential of being targets for treatment with PARP inhibitors. The recently described Poly ADP-Ribose Polymerase (PARP)-inhibitors have in several studies showed promising results in treating cancer in carriers of *BRCA1/2* variants known to affect function.[31–33]

Future development of the FFPE testing includes improving the design of the HaloPlex probes. Increasing the capture and amplification of smaller DNA fragments will improve the coverage especially in degraded DNA samples. Optimizing the DNA extraction could also improve the outcome, as higher DNA yield and concentration may lead to more usable DNA.

In conclusion, testing deceased persons for variants in *BRCA1/2,* using HaloPlex target enrichment and next-generation sequencing, is possible in archived FFPE tissue samples aged up to 30 years and may help to more accurately evaluate the risk of breast and ovarian cancer in some families, where genetic counseling otherwise would rely on risk assessment based on family history alone.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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