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TECHNICAL ADVANCE

Clinical Next-Generation Sequencing Pipeline Outperforms a Combined Approach Using Sanger Sequencing and Multiplex Ligation-Dependent Probe Amplification in Targeted Gene Panel Analysis

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Advances in next-generation sequencing (NGS) have facilitated parallel analysis of multiple genes enabling the implementation of cost-effective, rapid, and high-throughput methods for the molecular diagnosis of multiple genetic conditions, including the identification of *BRCA1* and *BRCA2* mutations in high-risk patients for hereditary breast and ovarian cancer. We clinically validated a NGS pipeline designed to replace Sanger sequencing and multiplex ligation-dependent probe amplification analysis and to facilitate detection of sequence and copy number alterations in a single test focusing on a *BRCA1/BRCA2* gene analysis panel. Our custom capture library covers 46 exons, including *BRCA1* exons 2, 3, and 5 to 24 and *BRCA2* exons 2 to 27, with 20 nucleotides of intronic regions both 5′ and 3′ of each exon. We analyzed 402 retrospective patients, with previous Sanger sequencing and multiplex ligation-dependent probe amplification results, and 240 clinical prospective patients. One-hundred eighty-three unique variants, including sequence and copy number variants, were detected in the retrospective ($n = 95$) and prospective ($n = 88$) cohorts. This standardized NGS pipeline demonstrated 100% sensitivity and 100% specificity, uniformity, and high-depth nucleotide coverage per sample (approximately 7000 reads per nucleotide). Subsequently, the NGS pipeline was applied to the analysis of larger gene panels, which have shown similar uniformity, sample-to-sample reproducibility in coverage distribution, and sensitivity and specificity for detection of sequence and copy number variants. *(J Mol Diagn 2016, 18: 657—667; http://dx.doi.org/10.1016/j.jmoldx.2016.04.002)*

Advances in the next-generation sequencing (NGS) technology have revolutionized clinical molecular diagnostics. Clinical utilization of massively parallel sequencing enables simultaneous assessment of multiple genes, gene panels, and entire exomes or genomes using a limited quantity of biological samples. Multiplexing of patient samples combined with broad genomic coverage results in significantly decreased cost and turnaround times (TATs).[1]

Mutations of highly penetrant cancer susceptibility genes, *BRCA1* and *BRCA2*, are the strongest genetic predisposition factors for hereditary breast and ovarian cancer (HBOC), increasing the lifetime risk of developing these cancers to as high as 80%.[2] Moreover, *BRCA1* and *BRCA2* mutations are also associated with earlier disease onset and with other types of cancers, including fallopian tube, gastric, pancreatic, and prostate cancers. The identification of *BRCA* mutations is important for individual and family genetic counseling, as well as for early implementation of

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personalized pharmacotherapy using poly (ADP-ribose) polymerase inhibitors.[3] Thus, the efficient identification of *BRCA1/2* mutation carriers among women affected with breast and/or ovarian cancers may be crucial for determining optimum therapeutic strategies for these patients and their affected family members.

Because of the relatively large size of the *BRCA1/2* genes and the allelic heterogeneity of the pathogenic mutations, clinical testing of these genes is technically complex and labor intensive and, as such, is currently restricted to screening high-risk patients as determined by their family cancer history or ethnic origin in the case of identified founder mutations.[4] Methods for mutation screening of the *BRCA1* and *BRCA2* genes have evolved since these genes were first characterized and testing was initiated in the mid-1990s.[5] The earliest approaches to *BRCA1/2* gene analysis involved indirect methods of mutation identification, such as protein truncation analysis which did not detect potentially damaging missense mutations or structural gene rearrangements. Subsequent improvements in mutation scanning approaches included denaturing high-performance liquid chromatography, and other methods such as multiplex ligation-dependent probe amplification (MLPA),[6,7] to help identify the small, although significant, proportion of potentially damaging genomic rearrangements in the *BRCA1* and *BRCA2* genes. Clinical molecular testing continued to evolve to Sanger sequencing[8] in conjunction with MLPA (currently considered the gold standard for sequencing assays), an approach that is highly sensitive but relatively expensive and labor intensive. Recent improvements to NGS technologies that facilitate massively parallel analysis of multiple genes have enabled the implementation of more cost-effective, rapid, and high-throughput methods to allow precise identification of *BRCA1* and *BRCA2* mutations in these high-risk patients, as well as testing of more comprehensive gene panels for cancer predisposition syndromes.[4,9,10] NGS is also capable of sensitive detection of sequence variants and may also be used for detection of copy number variants (CNVs), such as exon deletions and duplications that currently require use of MLPA or other copy number technologies.

Here, we describe the clinical validation of an NGS pipeline designed to replace Sanger sequencing and MLPA analysis and to facilitate highly sensitive and specific detection of sequence and copy number alterations in a single assay, using the *BRCA1/BRCA2* gene analysis panel as a test example, and to demonstrate application of this approach for analysis of other larger clinical gene panels.

Materials and Methods

Sample Collection

The clinical validation cohort (retrospective) comprised 104 HBOC patient specimens, previously tested using Sanger sequencing and MLPA method at the London Health Science Centre Molecular Genetics Laboratory, Molecular Diagnostics Division, and 298 HBOC patient samples, previously tested at the Hamilton Health Sciences Molecular Diagnostic Laboratory. We also analyzed a prospective cohort of the first 240 HBOC patients whose samples were initially tested with the NGS method at Hamilton Health Sciences; for those samples in which pathogenic, likely pathogenic, and variants of unknown clinical significance were identified, the findings were confirmed by Sanger sequencing and/or MLPA. NGS was performed at both institutions for their respective patients. All patients gave consent and were counseled for testing as part of their clinical HBOC screening.

DNA Isolation

Genomic DNA from each sample was isolated by standard protocols using the MagNA Pure system (Roche Diagnostics, Laval, QC, Canada) at London Health Sciences Centre, or the Qiagen Puregene method or QIAcube automated method (Qiagen, Hilden, Germany) at Hamilton Health Sciences. DNA was quantified by measuring absorbance with a DTX 880 Multimode Detector (Beckman Coulter, Brea, CA).

CNV Analysis Using MLPA

Genomic DNA (100 ng) was amplified according to the manufacturer's recommendations using a SALSA MLPA kit (P002-D1 and/or P087-C1 for *BRCA1*, P090-A4 and/or P077-A2 for *BRCA2*; MRC Holland, Amsterdam, the Netherlands). PCR products were separated by capillary electrophoresis on an ABI 3730 (Life Technologies, Thermo Fisher Scientific, Waltham, MA). Copy number alterations were analyzed with Coffalyser.Net software version 131211.1524 (MRC Holland).

Sanger-Based Sequencing

Coding regions and the flanking intronic regions (−20 bp to 10 bp) from the genes of interest were PCR-amplified and sequenced with the BigDye Terminator version 1.1 cycle sequencing kit (Life Technologies). Sequencing products were separated by capillary electrophoresis on an ABI 3730 (Life Technologies) and analyzed with Mutation Surveyor version 4.0.7 software (SoftGenetics, LLC, State College, PA).

NGS Library Design

Custom sequence capture probes were designed using the SeqCap EZ Choice Library system (Roche NimbleGen, Inc., Madison, WI). The design included enrichment for all coding exons as well as 20 bp of the 5′ and 3′ flanking intronic regions on each side. This design can include up to 2.1 million different probes that are designed to massively overlap each other across the target sequence, thereby

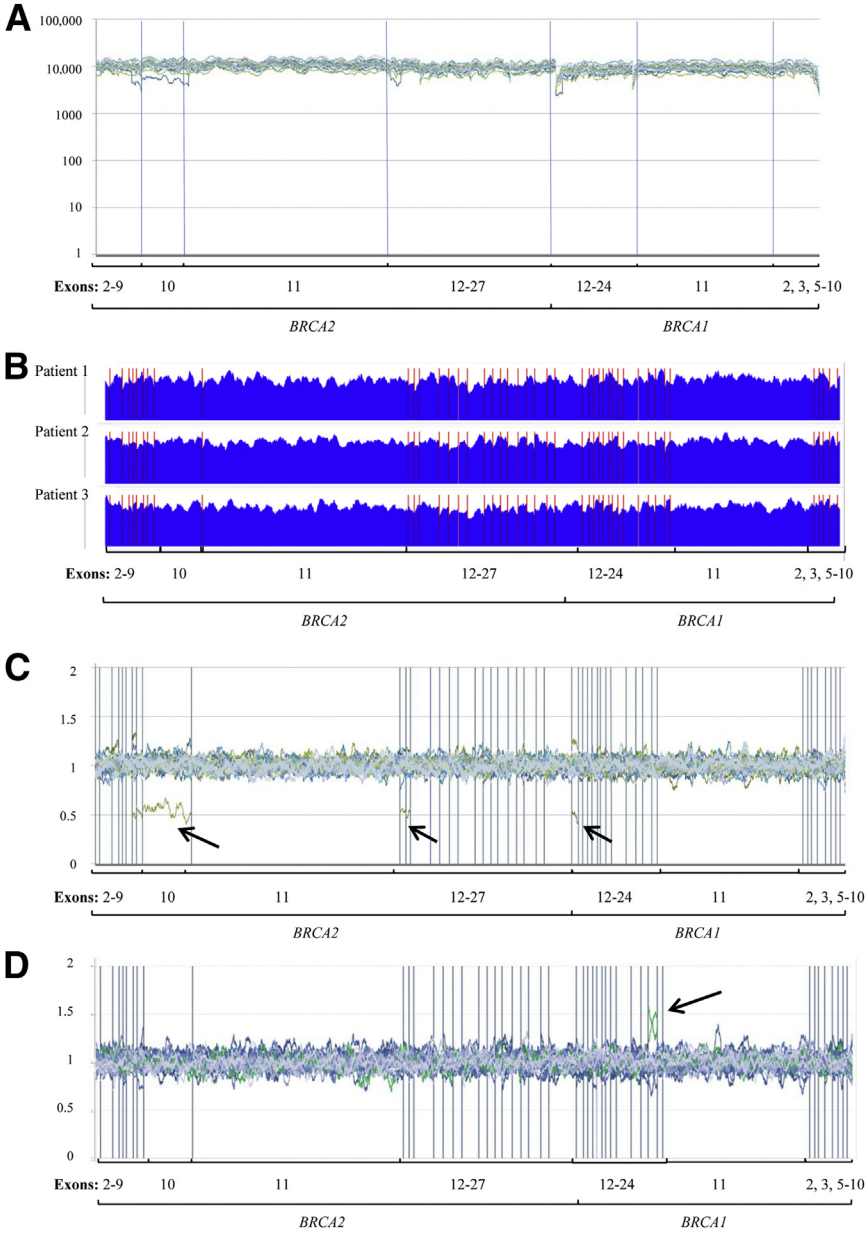

Figure 1 *BRCA1/2* gene panel. **A:** Coverage plot for a 24-patient sample batch (each line represents one patient); *y* axis is sequence read depth; gene and exon locations are indicated. **B:** Sequence coverage depth for three representative *BRCA1/2* screens; gene and exon locations are indicated; **red lines** indicate exon boundaries; *x* axis indicates nucleotide positions on corresponding genes and exons; *y* axis indicates depth of sequence coverage (approximate mean coverage for *BRACA1/2* = 7000); *y* axis is on a linear scale so for a sample with a mean coverage of 2000, reduction in coverage to the half equates to approximately 1000 times coverage at that locus. **C** and **D:** Normalized copy number plot demonstrating deletion detection (**arrows**, **C**) and gene duplication (**arrow**, **D**). Gene and exon locations are indicated (*x* axis); **blue lines** indicate exon boundaries; *y* axis represents quantile normalized copy number data (for autosomal genes 0.5 = one copy, 1 = two copies, 1.5 = three copies).

introducing significant redundancy and ability to capture complex, CG-rich, and polymorphic genomic regions. The SeqCap EZ Choice Library are proprietary designs involving Nimblegen-designed bioinformatically targeted probe coverage of the region of interest, that normally involves high-density tiling of the targeted region. If needed, each specific design can be adjusted for probe concentrations on empirical assessment on patient samples to ensure uniform depth of coverage.

Library Preparation and Target Capture Sequencing

Libraries were prepared with 100 ng of genomic DNA fragmented to 180 to 220 bp using a Covaris E220 Series Focused-ultrasonicator (Covaris, Inc., Woburn, MA). After fragment quantification and size distribution assessment with the Qubit fluorometer (Life Technologies) and 2200 TapeStation (Agilent Technologies, Santa Clara, CA), respectively, each sample library was ligated with a specific barcode index according to the manufacturer's protocol (Roche NimbleGen, Inc.). DNA libraries were then pooled and captured using the SeqCap EZ Choice Library system (Roche NimbleGen, Inc.). Captured libraries underwent appropriate quality control analysis and were diluted to a concentration of 4 nmol/L to process for sequencing according to the manufacturer's instructions (Illumina, San Diego, CA). The final captured library concentration for sequencing was 8 pmol/L with a 1% PhiX spike-in. Libraries were sequenced using the MiSeq version 2 reagent kit to generate 2 × 150 bp paired-end reads using the MiSeq fastq generation mode (Illumina), with 24 different patient samples multiplexed per run.

Table 1 *BRCA* Mutations Identified in the Retrospective Cohort ($n = 402$)

Gene	Patients, *n*	Variant type	cDNA change	Protein change	Homozygous/ heterozygous
BRCA1	1	Deletion	BRCA1:Ex1_24del	NA	0/1
BRCA1	1	Duplication	BRCA1:Ex13dup	NA	0/1
BRCA1	1	Duplication	BRCA1:Ex18_20dup	NA	0/1
BRCA1	23	Missense	c.1067A>G	p.Gln356Arg	0/23
BRCA1	1	Frame-shift	c.1174_1213del	p.Leu392Glnfs	0/1
BRCA1	1	Frame-shift	c.1386_1387insG	p.Lys463Glufs	0/1
BRCA1	1	Missense	c.1390A>G	p.Thr464Ala	0/1
BRCA1	1	Frame-shift	c.1500_1504del	p.Leu502Alafs	0/1
BRCA1	1	Nonsense	c.1687C>T	p.Gln563Ter	0/1
BRCA1	1	Frame-shift	c.1961delA	p.Lys654Serfs	0/1
BRCA1	1	Missense	c.2050C>T	p.Pro684Ser	0/1
BRCA1	23	Missense	c.2077G>A	p.Asp693Asn	1/22
BRCA1	102	Synonymous	c.2082C>T	p.Ser694=	22/80
BRCA1	101	Synonymous	c.2311T>C	p.Leu771=	20/81
BRCA1	1	Missense	c.2566T>C	p.Tyr856His	0/1
BRCA1	106	Missense	c.2612C>T	p.Pro871Leu	25/81
BRCA1	1	Synonymous	c.2733A>G	p.Gly911=	0/1
BRCA1	1	Missense	c.2791G>T	p.Val931Leu	0/1
BRCA1	1	Missense	c.3024G>A	p.Met1008Ile	0/1
BRCA1	1	Missense	c.3092T>G	p.Ile1031Ser	0/1
BRCA1	101	Missense	c.3113A>G	p.Glu1038Gly	20/81
BRCA1	8	Missense	c.3119G>A	p.Ser1040Asn	0/8
BRCA1	1	Frame-shift	c.3479_3489del	p.Glu1161Phefs	0/1
BRCA1	102	Missense	c.3548A>G	p.Lys1183Arg	20/82
BRCA1	3	Missense	c.4039A>G	p.Arg1347Gly	0/3
BRCA1	1	Synonymous	c.4050C>T	p.Gly1350=	0/1
BRCA1	101	Synonymous	c.4308T>C	p.Ser1436=	20/81
BRCA1	1	Synonymous	c.4416T>G	p.Leu1472=	0/1
BRCA1	1	Frame-shift	c.4417delT	p.Ser1473Leufs	0/1
BRCA1	1	Missense	c.4535G>T	p.Ser1512Ile	0/1
BRCA1	102	Missense	c.4837A>G	p.Ser1613Gly	20/82
BRCA1	1	Nonsense	c.4945A>T	p.Arg1649Ter	0/1
BRCA1	1	Missense	c.4946G>T	p.Arg1649Ile	0/1
BRCA1	1	Missense	c.4947A>T	p.Arg1649Ser	0/1
BRCA1	1	Frame-shift	c.4947delAins	p.Met1650Tyrfs	0/1
BRCA1	6	Missense	c.4956G>A	p.Met1652Ile	0/6
BRCA1	1	Missense	c.5207T>C	p.Val1736Ala	0/1
BRCA1	2	Frame-shift	c.5266_5267ins	p.Gln1756Profs	0/2
BRCA1	1	Synonymous	c.591C>T	p.Cys197=	0/1
BRCA1	1	Synonymous	c.981A>G	p.Thr327=	0/1
BRCA2	1	Deletion	BRCA1:Ex24del	NA	0/1
BRCA2	1	Deletion	BRCA2: Ex8_10del, 12_13del	NA	0/1
BRCA2	1	Deletion	BRCA2:Ex19_20del	NA	0/1
BRCA2	1	Synonymous	c.10095C>G	p.Val3365=	0/1
BRCA2	1	Frame-shift	c.10096_10097ins	p.Ser3366Asnfs	0/1
BRCA2	1	Missense	c.10234A>G	p.Ile3412Val	0/1
BRCA2	104	Missense	c.1114A>C	p.Asn372His	16/88
BRCA2	12	Synonymous	c.1365A>G	p.Ser455=	0/12
BRCA2	1	Missense	c.1487C>T	p.Ser496Phe	0/1
BRCA2	1	Missense	c.1762A>G	p.Asn588Asp	0/1
BRCA2	4	NA	c.1909+22_1909+23ins	NA	3/1
BRCA2	2	Synonymous	c.1938C>T	p.Ser646=	0/2
BRCA2	12	Synonymous	c.2229T>C	p.His743=	0/12
BRCA2	1	Synonymous	c.231T>G	p.Thr77=	0/1
BRCA2	1	Synonymous	c.2883G>A	p.Gln961=	0/1
BRCA2	12	Missense	c.2971A>G	p.Asn991Asp	0/12

(*table continues*)

Table 1 (*continued*)

Gene	Patients, *n*	Variant type	cDNA change	Protein change	Homozygous/ heterozygous
BRCA2	1	Missense	c.3262C>T	p.Pro1088Ser	0/1
BRCA2	92	Synonymous	c.3396A>G	p.Lys1132=	17/75
BRCA2	3	Synonymous	c.3516G>A	p.Ser1172=	0/3
BRCA2	62	Synonymous	c.3807T>C	p.Val1269=	8/54
BRCA2	2	Synonymous	c.4068G>A	p.Leu1356=	0/2
BRCA2	1	Missense	c.4094G>A	p.Cys1365Tyr	0/1
BRCA2	1	In-frame	c.4131_4132ins	p.Thr1378Ter	0/1
BRCA2	1	Missense	c.4163C>A	p.Thr1388Asn	0/1
BRCA2	1	Frame-shift	c.4169delT	p.Leu1390Trpfs	0/1
BRCA2	1	Missense	c.4189G>A	p.Glu1397Lys	0/1
BRCA2	3	Missense	c.4258G>T	p.Asp1420Tyr	0/3
BRCA2	191	Synonymous	c.4563A>G	p.Leu1521=	190/1
BRCA2	3	Synonymous	c.5199C>T	p.Ser1733=	0/3
BRCA2	6	Missense	c.5744C>T	p.Thr1915Met	0/6
BRCA2	1	Frame-shift	c.5775_5860del	p.Gln1925Hisfs	0/1
BRCA2	1	Missense	c.5869A>G	p.Ile1957Val	0/1
BRCA2	1	Missense	c.5945G>C	p.Ser1982Thr	0/1
BRCA2	1	Missense	c.6100C>T	p.Arg2034Cys	0/1
BRCA2	1	Frame-shift	c.6402_6406del	p.Asn2135Lysfs	0/1
BRCA2	191	Synonymous	c.6513G>C	p.Val2171=	190/1
BRCA2	1	Missense	c.6796A>C	p.Asn2266His	0/1
BRCA2	2	NA	c.68-7T>A	NA	0/2
BRCA2	1	Missense	c.7007G>A	p.Arg2336His	0/1
BRCA2	1	Missense	c.7010C>T	p.Thr2337Ile	0/1
BRCA2	70	Synonymous	c.7242A>G	p.Ser2414=	7/63
BRCA2	191	Missense	c.7397T>C	p.Val2466Ala	190/1
BRCA2	2	Missense	c.7469T>C	p.Ile2490Thr	0/2
BRCA2	143	NA	c.7806-14T>C	NA	50/93
BRCA2	1	Missense	c.8149G>T	p.Ala2717Ser	0/1
BRCA2	1	Missense	c.8182G>A	p.Val2728Ile	0/1
BRCA2	12	Missense	c.865A>C	p.Asn289His	0/12
BRCA2	1	Missense	c.8567A>C	p.Glu2856Ala	0/1
BRCA2	1	Synonymous	c.8460A>C	p.Val2820=	0/1
BRCA2	1	Frame-shift	c.8537_8538del	p.Glu2846Glyfs	0/1
BRCA2	1	Synonymous	c.9234C>T	p.Val3078=	0/1
BRCA2	3	NA	c.9257-16T>C	NA	0/3
BRCA2	1	Frame-shift	c.9403delC	p.Leu3135Phefs	0/1
BRCA2	1	NA	c.9649-19G>A	NA	0/1
BRCA2	3	Nonsense	c.9976A>T	p.Lys3326Ter	0/3

NA, not applicable.

NGS Analysis for Sequence Variants

Sequence analysis for variant identification, alignment, and coverage distribution was performed with NextGene software version 2.4.1 (SoftGenetics, LLC) using standard alignment settings (allowable mismatch bases: 1; allowable ambiguous alignments: 50; seeds bases: 30; move step bases: 5; allowable alignments: 100; matching base percentage >85%). BAM and VCF files were imported into Geneticist Assistant version 1.1.5 (SoftGenetics, LLC) for quality control assessment (minimum base coverage; mean exon coverage) and for data basing. Variants were analyzed and interpreted by a clinical molecular geneticist and were classified for pathogenicity using the American College of Medical Genetics guidelines.[11]

NGS Analysis of CNVs

Reports for base coverage distribution were generated using NextGene software version 2.4.1 (SoftGenetics, LLC). Single nucleotide coverage for each patient was normalized. Briefly, the sum of all 24 sample sequencing reads divided by the number of patients equals the total mean coverage per patient (mean of sums). The normalization factor (NF) is then calculated by dividing the sum of reads for each patient by the mean of sums. Finally, each read per nucleotide per patient is divided by the NF and by the average read of each nucleotide in the 24 samples, resulting in the normalized reads per nucleotide per patient (NR). The normalized data were then presented in a graph allowing visualization of CNVs

(ie, deletions and duplications) at exon and subexon levels (Excel version 14.0.6129.5000; Microsoft Corporation, Redmond, WA). Deletions were defined by a mean ratio of ≤0.65 and duplications were defined by a ratio of ≥1.35.

$$\frac{\sum Reads}{\#Patients} = Mean\ of\ Sums \quad (1)$$

$$\frac{\sum Reads\ per\ individual\ patient}{Mean\ of\ Sums} = NF \quad (2)$$

$$\frac{Reads\ per\ nucleotide\ per\ patient}{NF * Average\ Reads\ per\ nucleotide} = NR \quad (3)$$

Results

Sequencing Coverage and Normalization

Our custom capture library covers 46 exons, including *BRCA1* exons 2, 3, and 5 to 24 and *BRCA2* exons 2 to 27, with 20 nucleotides of intronic regions both 5′ and 3′ of each exon, with a total of 17,769 nucleotides (89% in coding regions and 11% in flanking regions). Sequencing *BRCA1* and *BRCA2* in the retrospective cohort generated a mean of approximately 6500 reads per nucleotide per patient, with 24 patient samples batched per library enrichment and sequencing run. The raw sequence alignment of 24 patients in a single run is shown in Figure 1A, demonstrating deep and uniform coverage across all nucleotides analyzed. In addition, the high level of uniformity of coverage among patients is evident at the subexon resolution (Figure 1B). Taking advantage of the reproducibility and uniformity of the coverage distribution, we applied a quantile normalization algorithm to the coverage distribution reports from the NextGene alignment output for each patient to assess copy number alterations. Figure 1C shows an example of a quantile normalized copy number graph for a 24-patient batch that included a patient with a *BRCA1* exon 24 deletion and another patient with a complex *BRCA2* deletion involving exons 8 to 10 and 12 to 13, with normal exon 11 copy number. Similarly, two patients' samples with a *BRCA1* duplication involving exon 13 demonstrate an easily detectable, distinct abnormality on the copy number plot (Figure 1D).

BRCA1 and *BRCA2* Variants

NGS analysis for *BRCA1* and *BRCA2* was performed in a retrospective cohort of 402 HBOC individuals that had previously been analyzed by Sanger sequencing and MLPA techniques. A summary of the sequence variants is shown in Table 1. The retrospective cohort are patients who were previously tested by the Sanger sequencing and MLPA method and included preselected patients with the wide range of the types of mutations found in these genes, as well as select normal controls. The information for pathogenic, variants of unknown clinical significance (VUSs), and benign variants was available for all patients and was compared with the NGS data. Briefly, we identified 95 different variants (40 in *BRCA1*, 55 in *BRCA2*), resulting in a total of 1964 variant occurrences. To further characterize the variant frequency distribution, they (both sequence and CNVs) were classified into seven groups: intronic heterozygous single nucleotide polymorphisms (SNPs), intronic homozygous SNPs, coding heterozygous SNPs, coding homozygous SNPs, deletions, insertions, and exon deletions/duplications. We observed 1022 (52%) heterozygous SNPs and 763 (39%) homozygous SNPs in coding regions, as well as 153 (7.8%) intronic SNPs. In addition, among the 1964 variants, 26 (1.32%) were CNVs that included 20 deletions and insertions and six exon del/dups (Figure 2A). All previously observed variants were confirmed, demonstrating 100% sensitivity of the NGS technology. Five additional variants outside of the region included in the original Sanger sequencing were also detected, and these variants were subsequently confirmed by Sanger sequencing.

The prospective cohort included select patients in whom pathogenic variants or VUSs were detected by the NGS assay and were subsequently confirmed by Sanger sequencing and MLPA, as well as normal controls. In the prospective cohort of 240 HBOC patients tested by NGS,

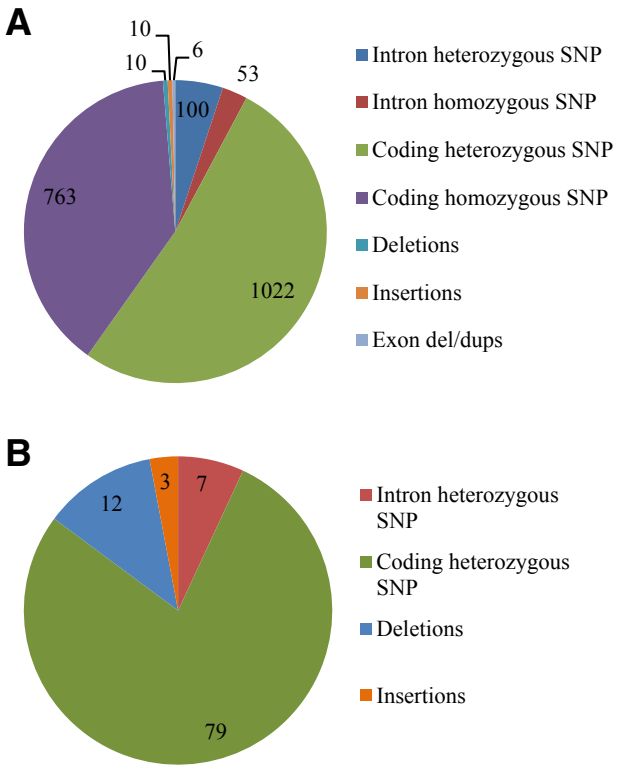

Figure 2 **A:** *BRCA1* and *BRCA2* variants identified by NGS in the retrospective cohort. SNPs were classified according to zygosity (homozygous/heterozygous) and location (intron or exon). **B:** Type of *BRCA1* and *BRCA2* variants identified by NGS in the prospective patient cohort. The number of deletions, insertions, and exon deletion/duplications identified are shown in each panel. $n = 402$ (**A**); $n = 240$ (**B**). del, deletion; dup, duplication; NGS, next-generation sequencing; SNP, single nucleotide polymorphism.

Table 2 *BRCA* Mutations Identified in the Prospective Cohort ($n = 240$)

Gene	Patients, *n*	Variant type	cDNA change	Protein change	Zygosity
BRCA1	1	Synonymous	c.1065G>A	p.Lys355=	HZ
BRCA1	1	Nonsense	c.1154G>A	p.Trp385Ter	HZ
BRCA1	1	Missense	c.1487G>A	p.Arg496His	HZ
BRCA1	1	Missense	c.1745C>T	p.Thr582Met	HZ
BRCA1	1	Missense	c.181T>G	p.Cys61Gly	HZ
BRCA1	2	Synonymous	c.1971A>G	p.Gln657=	HZ
BRCA1	1	Missense	c.2566T>C	p.Tyr856His	HZ
BRCA1	1	Missense	c.2596C>T	p.Arg866Cys	HZ
BRCA1	1	Frame-shift	c.2681_2682del	p.Lys894Thrfs	HZ
BRCA1	1	Missense	c.2834G>C	p.Ser945Thr	HZ
BRCA1	1	Frame-shift	c.2835_2836del	p.Ile946Glnfs	HZ
BRCA1	1	Missense	c.3082C>T	p.Arg1028Cys	HZ
BRCA1	1	Synonymous	c.3270A>G	p.Gln1090=	HZ
BRCA1	1	Nonsense	c.3400G>T	p.Glu1134Ter	HZ
BRCA1	1	Missense	c.3560G>A	p.Ser1187Asn	HZ
BRCA1	1	Missense	c.3600G>C	p.Gln1200His	HZ
BRCA1	1	Missense	c.3608G>A	p.Arg1203Gln	HZ
BRCA1	1	Missense	c.3784T>C	p.Ser1262Pro	HZ
BRCA1	1	Nonsense	c.4480G>T	p.Glu1494Ter	HZ
BRCA1	1	Missense	c.4771G>A	p.Gly1591Ser	HZ
BRCA1	1	Synonymous	c.4812A>G	p.Gln1604=	HZ
BRCA1	1	Nonsense	c.5136G>A	p.Trp1712Ter	HZ
BRCA1	1	Frame-shift	c.514delC	p.Gln172Asnfs	HZ
BRCA1	1	Synonymous	c.5157G>T	p.Val1719=	HZ
BRCA1	2	Frame-shift	c.5266_5267ins	p.Gln1756Profs	HZ
BRCA1	1	Missense	c.5324T>G	p.Met1775Arg	HZ
BRCA1	1	N/A	c.5332+13G>T	N/A	HZ
BRCA1	1	Synonymous	c.591C>T	p.Cys197=	HZ
BRCA1	2	N/A	c.81-14C>T	N/A	HZ
BRCA1	1	Nonsense	c.962G>A	p.Trp321Ter	HZ
BRCA2	1	Synonymous	c.10110G>A	p.Arg3370=	HZ
BRCA2	1	Missense	c.10111A>G	p.Thr3371Ala	HZ
BRCA2	2	Missense	c.1151C>T	p.Ser384Phe	HZ
BRCA2	1	Missense	c.125A>G	p.Tyr42Cys	HZ
BRCA2	1	Synonymous	c.1362A>G	p.Lys454=	HZ
BRCA2	1	Nonsense	c.1528G>T	p.Glu510Ter	HZ
BRCA2	1	Synonymous	c.183A>G	p.Leu61=	HZ
BRCA2	1	N/A	c.1909+22_1909+23ins	N/A	HZ
BRCA2	1	Missense	c.223G>C	p.Ala75Pro	HZ
BRCA2	1	Synonymous	c.2538A>C	p.Ser846=	HZ
BRCA2	1	Frame-shift	c.26delC	p.Pro9Glnfs	HZ
BRCA2	1	Missense	c.2803G>A	p.Asp935Asn	HZ
BRCA2	1	Synonymous	c.2919G>A	p.Ser973=	HZ
BRCA2	2	Missense	c.2926T>A	p.Ser976Thr	HZ
BRCA2	2	Missense	c.2927C>T	p.Ser976Phe	HZ
BRCA2	1	Missense	c.3055C>G	p.Leu1019Val	HZ
BRCA2	1	Frame-shift	c.3170_3174del	p.Lys1057Thrfs	HZ
BRCA2	1	Synonymous	c.3264T>C	p.Pro1088=	HZ
BRCA2	1	Missense	c.3445A>G	p.Met1149Val	HZ
BRCA2	2	Synonymous	c.4068G>A	p.Leu1356=	HZ
BRCA2	1	Synonymous	c.4434A>G	p.Leu1478=	HZ
BRCA2	1	Missense	c.4585G>A	p.Gly1529Arg	HZ
BRCA2	1	Missense	c.4681C>A	p.His1561Asn	HZ
BRCA2	2	Synonymous	c.4686A>G	p.Gln1562=	HZ
BRCA2	1	Nonsense	c.4965C>G	p.Tyr1655Ter	HZ
BRCA2	1	Synonymous	c.5418A>G	p.Glu1806=	HZ
BRCA2	1	Missense	c.5552T>G	p.Ile1851Ser	HZ
BRCA2	2	Missense	c.5704G>A	p.Asp1902Asn	HZ
BRCA2	1	Frame-shift	c.5722_5723del	p.Leu1908Argfs	HZ
BRCA2	1	Nonsense	c.5857G>T	p.Glu1953Ter	HZ
BRCA2	1	Missense	c.6322C>T	p.Arg2108Cys	HZ
BRCA2	3	Missense	c.6323G>A	p.Arg2108His	HZ
BRCA2	1	Missense	c.6325G>A	p.Val2109Ile	HZ
BRCA2	1	Missense	c.6412G>T	p.Val2138Phe	HZ
BRCA2	1	Frame-shift	c.6486_6489del	p.Lys2162Asnfs	HZ
BRCA2	1	Frame-shift	c.658_659delGT	p.Val220Ilefs	HZ
BRCA2	1	N/A	c.68-7T>A	N/A	HZ
BRCA2	1	Synonymous	c.6987G>A	p.Pro2329=	HZ
BRCA2	1	Missense	c.7017G>C	p.Lys2339Asn	HZ
BRCA2	1	Frame-shift	c.7069_7070del	p.Leu2357Valfs	HZ
BRCA2	1	Missense	c.7319A>G	p.His2440Arg	HZ
BRCA2	1	Missense	c.7544C>T	p.Thr2515Ile	HZ
BRCA2	1	Frame-shift	c.7577delC	p.Ala2526Glufs	HZ
BRCA2	1	Frame-shift	c.7580delT	p.Val2527Glufs	HZ
BRCA2	1	Frame-shift	c.7583delG	p.Gly2528Glufs	HZ
BRCA2	1	N/A	c.7805+6C>G	N/A	HZ
BRCA2	1	Missense	c.7916C>T	p.Pro2639Leu	HZ
BRCA2	1	Missense	c.8057T>G	p.Leu2686Arg	HZ
BRCA2	1	Missense	c.825A>T	p.Lys275Asn	HZ
BRCA2	1	Missense	c.8356G>A	p.Ala2786Thr	HZ
BRCA2	1	N/A	c.8487+3A>G	N/A	HZ
BRCA2	3	Missense	c.8567A>C	p.Glu2856Ala	HZ
BRCA2	1	N/A	c.8633-16C>G	N/A	HZ
BRCA2	1	Missense	c.9032T>C	p.Leu3011Pro	HZ
BRCA2	1	Missense	c.9038C>T	p.Thr3013Ile	HZ
BRCA2	1	Missense	c.9076C>G	p.Gln3026Glu	HZ
BRCA2	1	Missense	c.9271G>A	p.Val3091Ile	HZ
BRCA2	1	Missense	c.9730G>A	p.Val3244Ile	HZ

HZ, heterozygous.

88 different sequence variants (30 in *BRCA1* and 58 in *BRCA2*) (Table 2, Figure 2B) classified as pathogenic, likely pathogenic, or VUSs were observed. One hundred one *BRCA1* and *BRCA2* variant occurrences were observed, of which 93 (79 sequence variants, 12 deletions, and 2 insertion) were in coding regions and eight (seven sequence variants and one insertion) were intronic. All variants were confirmed by Sanger sequencing and MLPA analysis, demonstrating 100% specificity for the NGS analysis.

Discussion

In this study, we present clinical validation of a NGS pipeline designed to replace conventional Sanger sequencing and MLPA for detection of sequence and CNVs, respectively. NGS-based analysis of the *BRCA1* and *BRCA2* genes were previously reported, including PCR-based library preparation on the Roche sequencer,[10] PCR-based on Ion-Torrent sequencer,[4,12] PCR-based on SOLiD and Ion−Torrent sequencers,[13] and RainDance microdroplet

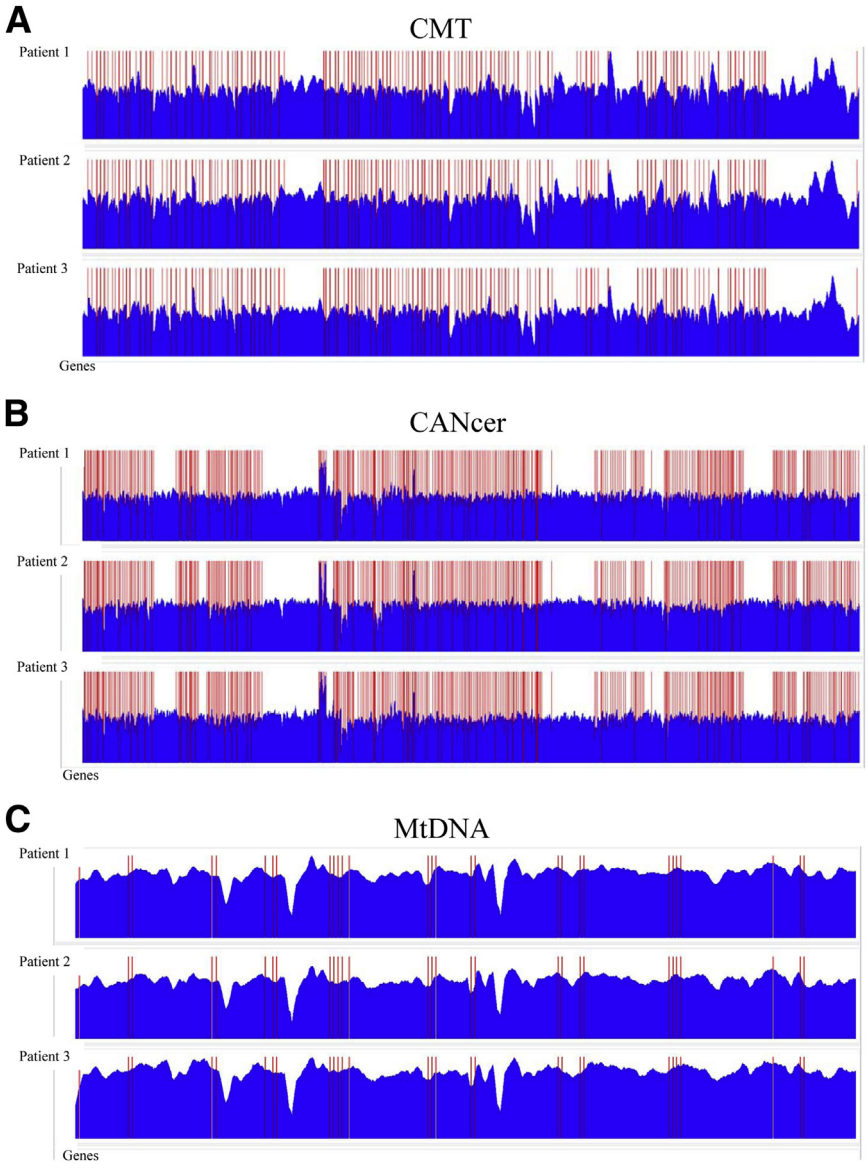

Figure 3 Sequence coverage depth for three representative patient screens for CMT panel (17 genes: *MFN2*, *LMNA*, *MPZ*, *RAB7A*, *SH3TC2*, *FIG4*, *GARS*, *HSPB1*, *NEFL*, *GDAP1*, *EGR2*, *TRPV4*, *HSPB8*, *LITAF*, *PMP22*, *PRX*, *GJB1*) (**A**); CANcer (25 genes: *MUTYH*, *EPCAM*, *MSH2*, *MSH6*, *BARD1*, *MLH1*, *APC*, *PMS2*, *NBN*, *CDKN2A*, *BMPR1A*, *PTEN*, *ATM*, *CDK4*, *BRCA2*, *PALB2*, *CDH1*, *TP53*, *RAD51D*, *BRCA1*, *RAD51C*, *BRIP1*, *SMAD4*, *STK11*, *CHEK2*) (**B**); and MtDNA panel (37 genes: *ATP6*, *ATP8*, *COX1*, *COX2*, *COX3*, *CYTB*, *ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5*, *ND6*, *TRNA*, *TRNC*, *TRND*, *TRNE*, *TRNF*, *TRNG*, *TRNH*, *TRNI*, *TRNK*, *TRNL1*, *TRNL2*, *TRNM*, *TRNN*, *TRNP*, *TRNQ*, *TRNR*, *TRNS1*, *TRNS2*, *TRNT*, *TRNV*, *TRNW*, *TRNY*, *RNR1*, *RNR2*) (**C**). **Red lines** indicate exon boundaries. The *x* axis indicates nucleotide positions on corresponding genes and exons; *y* axis indicates depth of sequence coverage (approximate mean coverage for CMT, 2000; CANcer panel, 4000, and MtDNA, 5000 and 20,000 for blood and muscle, respectively). The *y* axis is on a linear scale so for a sample with a mean coverage of 2000, reduction in coverage to the half equates to approximately 1000 times coverage at that locus. CANcer, hereditary cancer; CMT, Charcot-Marie Tooth; MtDNA, mitochondrial genome sequencing.

PCR-based sequencing.[14] Although many of the approaches describe ability to sensitively detect sequence-level variation (SNPs and small insertions and deletions), all of these assays require parallel methods for the exon-level copy number detection. Our method is unique in its ability to use NGS data to sensitively detect both sequence level and copy number alterations in the same assay.

The key variables to be assessed when validating a clinical assay are sensitivity and specificity. We conducted NGS analysis of samples from 402 individuals, in which Sanger sequencing and MLPA testing had been conducted as part of the routine clinical workup. A total of 1964 variants, including coding and intronic SNPs, deletions, insertions, and exon level copy number alterations (Figure 2A, Table 1), were validated with 100% sensitivity. As part of the clinical laboratory standard operating procedures, reportable pathogenic variants and VUSs were confirmed from the source DNA specimen using an alternate technique (Sanger sequencing, MLPA, long-range PCR, etc.) to confirm the mutation and sample fidelity. Each of the 88 variants detected in the first 240 clinical NGS analyses was verified by confirmatory testing, demonstrating 100% specificity of the NGS assay.

One of the limitations of both Sanger sequencing and MLPA analysis relates to allele dropout, which pertains to the inability of a PCR primer, sequencing primer, or an MLPA probe to bind and subsequently amplify one of the two alleles that may contain a sequence variant or a polymorphism,[15,16] thereby resulting in the reduced sensitivity. Allele dropout is effectively abolished in this NGS pipeline due to the redundancy that is introduced by the Roche/Nimblegen library preparation protocol, including random genomic fragmentation and densely overlapped DNA library capture probes (up to two million unique probes per design). In fact, one of the patient samples in the prospective cohort with an MLPA-detected exon copy number loss,

which subsequently could not be confirmed by NGS analysis, was demonstrated to exhibit a SNP at the MLPA probe binding site, resulting in a false-positive MLPA result. Thus, the results presented herein demonstrate that this NGS pipeline outperforms at all levels the current gold standard of combined Sanger sequencing and MLPA.

A potential limitation of NGS-based assays can be a reduced ability to detect CNVs and complex sequence variants.[17] Our targeted NGS analytical pipeline takes advantage of the high depth of sequence coverage (mean depth of 2000 to 10,000 times, depending on the panel and number of patients) and intersample coverage uniformity (Figures 1B and 3) to address those limitations. Unlike off-the-shelf generic panel design, or even exome-slices where larger gene sets are sequenced and only genes of interest are analyzed and reported, targeted panel designs provide a significantly greater depth of coverage. With the use of a single nucleotide resolution depth of coverage information from the coverage distribution files generated by the NextGene alignment software and a quantile normalization algorithm, we are able to detect with 100% sensitivity and specificity the copy number alterations for each individual exon and all genes in our previously defined retrospective samples in every panel examined (see the description of other panels in the following paragraphs). In addition to replacing the MLPA screening in the *BRCA1/2* panel, we no longer perform MLPA testing as a primary screen for any gene panel.

Another aspect related to a specifically targeted panel design is scalability. Depending on the sample volumes and the required TAT, there is significant opportunity for scaling up both patient sample number and gene number. The abundance and redundancy in our probe designs makes it relatively easy to introduce additional genes to a design without significantly compromising the depth of coverage and increasing the possibility of sequence dropouts. Targeted gene panel can be designed to yield complete coverage for every single nucleotide in every gene with minimal sample-to-sample variability (Figures 1B and 3), eliminating the necessity for Sanger sequence back-filling, a common complication with clinical NGS tests with lower levels of coverage, or PCR-based enrichment designs. Therefore, this NGS pipeline allows for extensive scalability both in patient volume and gene number.

Although providing significant improvements in test quality, sensitivity, and specificity compared with standard NGS approaches as well as the gold standard Sanger sequencing/MLPA, this NGS pipeline also provides significant cost advantages through both reduced reagent costs and labor time. With the use of the simplest *BRCA1/2* two-gene panel with 24 patients per MiSeq run compared with the classic Sanger sequencing and MLPA testing, we can achieve approximately 70% reduction in total test costs for reagents, labor, and significant reduction in TAT (including service contracts and institutional overhead). Cost-effectiveness can be enhanced further through increased automation and scalability (100 patients per run instead of 24). If, however, a laboratory does not have the sufficient volumes to batch 24 or more samples per run, it is possible to combine multiple lower volume test panels on the same enrichment library design. This would ensure cost-effectiveness while enabling low TATs. Similar advantages are achieved in expanded, comprehensive gene panels (eg, CANcer panel, described in the following paragraphs), enabling significant improvements in efficiency of delivery of patient care and genetic services with a net positive financial impact on the health care system.

In addition to the *BRCA1/2* panel, this NGS pipeline has been applied to the analysis of other larger gene panels, including Charcot Marie Tooth (CMT) syndrome panel, hereditary cancer syndrome panel, and mitochondrial genome sequencing panel (Figure 3, Table 3) with similar results. One hundred percent sensitivity and specificity for detection of CNVs was also achieved for these panels (Figure 4). For example, our hereditary cancer panel design totaling 25 genes and approximately 100 kb of targeted sequence, when performed in a batch of 24 patients on a single MiSeq (2 × 150 chip) run, achieves a mean of

Table 3 Genes Included in Charcot Marie Tooth Panel (CMT; 17 Genes) Hereditary Cancer Panel (CANcer; 25 Genes), and Mitochondrial Genome Sequencing Panel (MtDNA; 37 Genes)

Panel	Genes		
CMT	*MFN2*	*GDAP1*	*HSPB8*
	LMNA	*EGR2*	*LITAF*
	MPZ	*TRPV4*	*PMP22*
	RAB7A	*GARS*	*PRX*
	SH3TC2	*HSPB1*	*GJB1*
	FIG4	*NEFL*	
CANcer	*MUTYH*	*CDK4*	*NBN*
	EPCAM	*BRCA2*	*CDKN2A*
	MSH2	*PALB2*	*BMPR1A*
	MSH6	*CDH1*	*PTEN*
	BARD1	*TP53*	*ATM*
	MLH1	*RAD51D*	*BRIP1*
	APC	*BRCA1*	*SMAD4*
	PMS2	*RAD51C*	*STK11*
	CHEK2		
	Genes	tRNAs	
MtDNA	*ATP6*	*TRNA*	*TRNP*
	ATP8	*TRNC*	*TRNQ*
	COX1	*TRND*	*TRNR*
	COX2	*TRNE*	*TRNS1*
	COX3	*TRNF*	*TRNS2*
	CYTB	*TRNG*	*TRNT*
	ND1	*TRNH*	*TRNV*
	ND2	*TRNI*	*TRNW*
	ND3	*TRNK*	*TRNY*
	ND4	*TRNL1*	rRNAs
	ND4L	*TRNL2*	*RNR1*
	ND5	*TRNM*	*RNR2*
	ND6	*TRNN*	

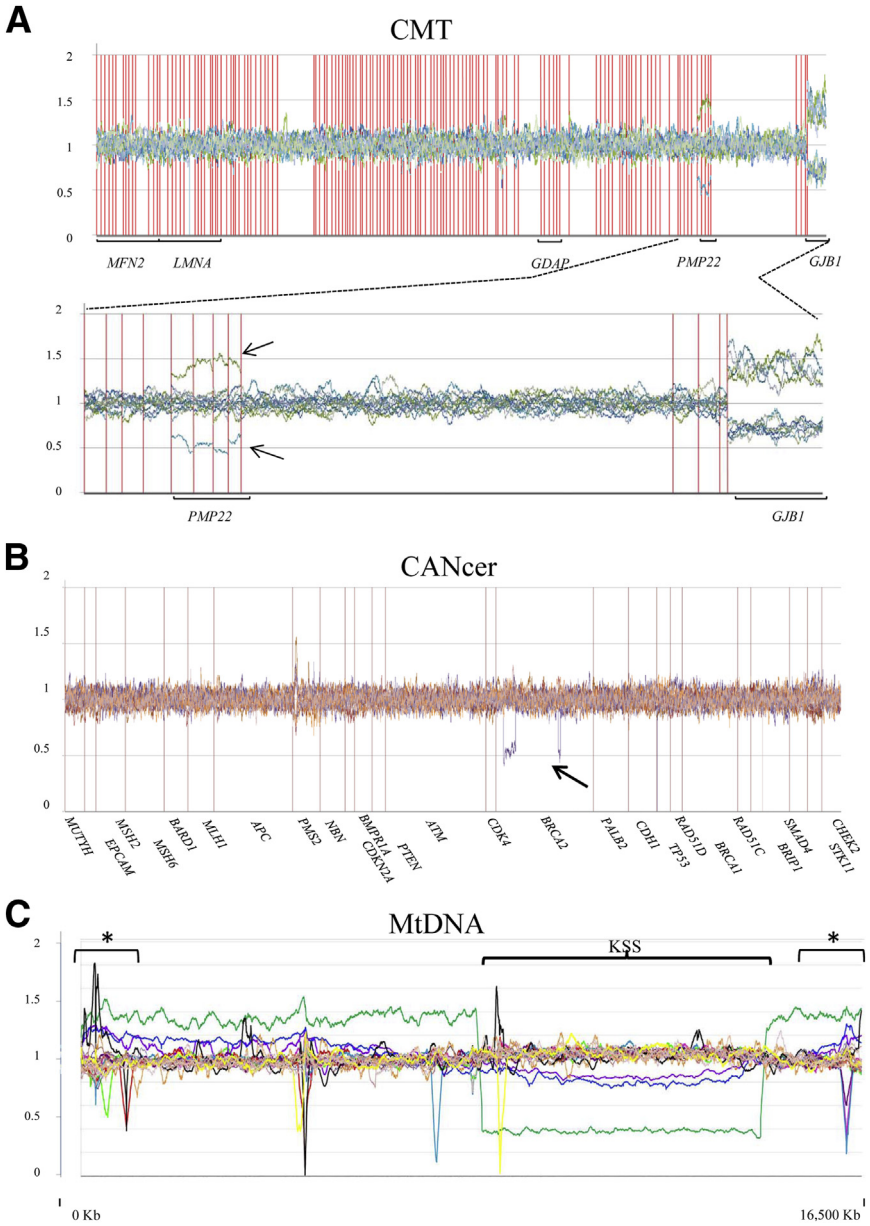

Figure 4 **A:** Normalized copy number plots for CMT; **top image** shows the comprehensive CMT panel; **bottom image** is a zoom in of *PMP22* gene showing detection of one deletion and one duplication (indicated by **arrows**). **Red lines** indicate exon boundaries. **B:** CANcer panel; **arrow** indicates a patient with complex multi-exon deletion in *BRCA2* (same patient as in Figure 1C). **Red lines** indicate exon boundaries. **C:** MtDNA panel; identification of patients with KSS, with approximately 10% heteroplasmy (in blue and pink) and approximately 50% heteroplasmy (in green). **Asterisks** indicate hypervariable D-loop regions (m.16,024 to m.576). **A**–**C:** The *x* axis indicates gene/exon positions; *y* axis represents quantile normalized copy number data (for autosomal genes 0.5 = one copy, 1 = two copies, 1.5 = three copies). CANcer, hereditary cancer; CMT, Charcot-Marie Tooth; KSS, Kearns-Sayre syndrome; MtDNA, mitochondrial genome sequencing.

4000 times depth of coverage, with no single nucleotide covered <500 times (Figures 3B and 4B). This approach has also enabled detection of the most common mutations in CMT (a whole gene deletion or duplication of the *PMP22* gene resulting in the hereditary neuropathy with pressure palsies or CMT1, respectively), which previously required stand-alone MLPA prescreening of all patients with suspected CMT or hereditary neuropathy with pressure palsies before the sequencing analysis (Figure 4A). Furthermore, our mitochondrial genome sequencing panel demonstrates the ability to sensitively detect the common 5-kb deletion associated with the Kearns-Sayre syndrome in peripheral blood samples of patients with as little as 10% heteroplasmy (Figure 4C), along with sensitive detection of sequence variants down to 2% heteroplasmy. Importantly, this NGS pipeline design ensures that any copy number alterations and/or complex sequence alterations that result in disrupted NGS sequence alignment and loss of sequence coverage would be detectable on the copy number plot, triggering further investigation and confirmation, thereby ensuring absolute sensitivity for complex sequence and CNVs.

Although screening entire genomes using NGS technology may be warranted for some clinical indications[18,19] and for genetic research and gene discovery, targeted gene panel sequencing provides the optimal way to deliver clinically significant, high-quality, and cost-effective genetic diagnostic. Such targeted panel-based genetic testing, in contrast to the higher cost and lower sensitivity/specificity of genome screening or exome-slicing, remains the preferred choice for polygenic conditions in which identification of a genetic defect affects patient management and clinical care. Here, we have described a clinical NGS pipeline that

outperforms the current gold standard Sanger sequencing and MLPA. Given the promise of the NGS technology to further expand the ability and capacity to discover and test an ever expanding number of genes, it is important to ensure that the application of NGS in a clinical setting does not happen at the cost of lowering the clinical standards of test delivery. Rather, when displacing a gold standard technology, we should aim to do it with a platinum standard one, while keeping the focus on clinical utility, patient care, and fiscal responsibility.

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