CLINICAL NGS PIPELINE OUTPERFORMS A COMBINED APPROACH USING SANGER SEQUENCING AND MLPA IN TARGETED GENE PANEL ANALYSIS

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Introduction

Recent advances in next-generation sequencing (NGS) have enabled parallel analysis of multiple genes allowing the implementation of more cost-effective, rapid and high-throughput methodologies for effective identification of single nucleotide variants (SNVs) and copy number variants (CNVs).

The objective of this study was to develop a single NGS-based pipeline to replace clinical Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA) copy number analysis, first for hereditary breast and ovarian cancer (HBOC) testing (BRACA1&2 panel), and then for other clinical gene panel tests, including CANcer, mitochondrial DNA (mtDNA), Charcot Marie Tooth (CMT), Assorted, lysosomal storage disease/urea cycle disorder, Dyslipidemia, Epilepsy and Hyperferritinemia.

Methods

BRACA1&2 validation panel: 402 retrospective patients, with previous Sanger and MLPA confirmation of HBOC, and 240 clinical prospective patients.

Additional panels: 391 retrospective patient and 2375 prospective patient.

Custom sequence capture probes were designed using the SeqCap EZ Choice Library system. NGS was conducted using an Illumina MiSeq platform with 12 or 24 different patient samples multiplexed per run. SNV identification, alignment and coverage distribution was performed with NextGene software v2.4.1, and CNV analysis was performed using single nucleotide resolution depth of coverage and a quantile normalization algorithm.
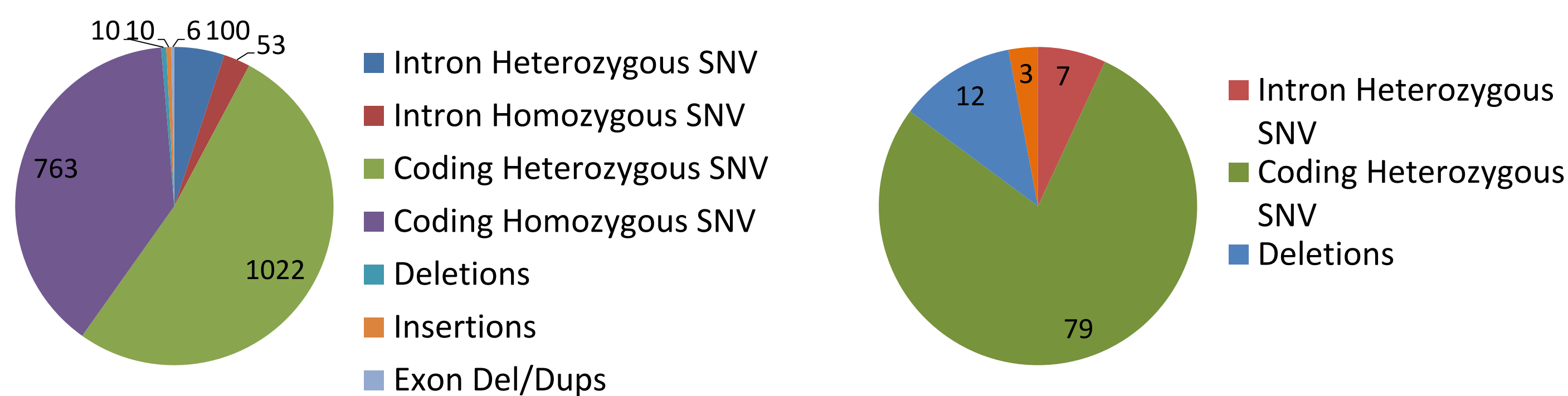
Clinical prospective patients identified with pathogenic, likely pathogenic, and variants of unknown clinical significance were confirmed by a second technique.

Results

BRACA1&2 validation panel: variants identified by NGS (Figure 1).

Retrospective (1964 variants) — Intron Heterozygous SNV, Intron Homozygous SNV, Coding Heterozygous SNV, Coding Homozygous SNV, Deletions, Insertions, Exon Del/Dups

Prospective (88 variants) — Intron Heterozygous SNV, Coding Heterozygous SNV, Deletions

Additional panels: All NGS panels demonstrated uniformity and high depth nucleotide coverage per sample, as well as ability to accurately detect CNV (Figure 2).
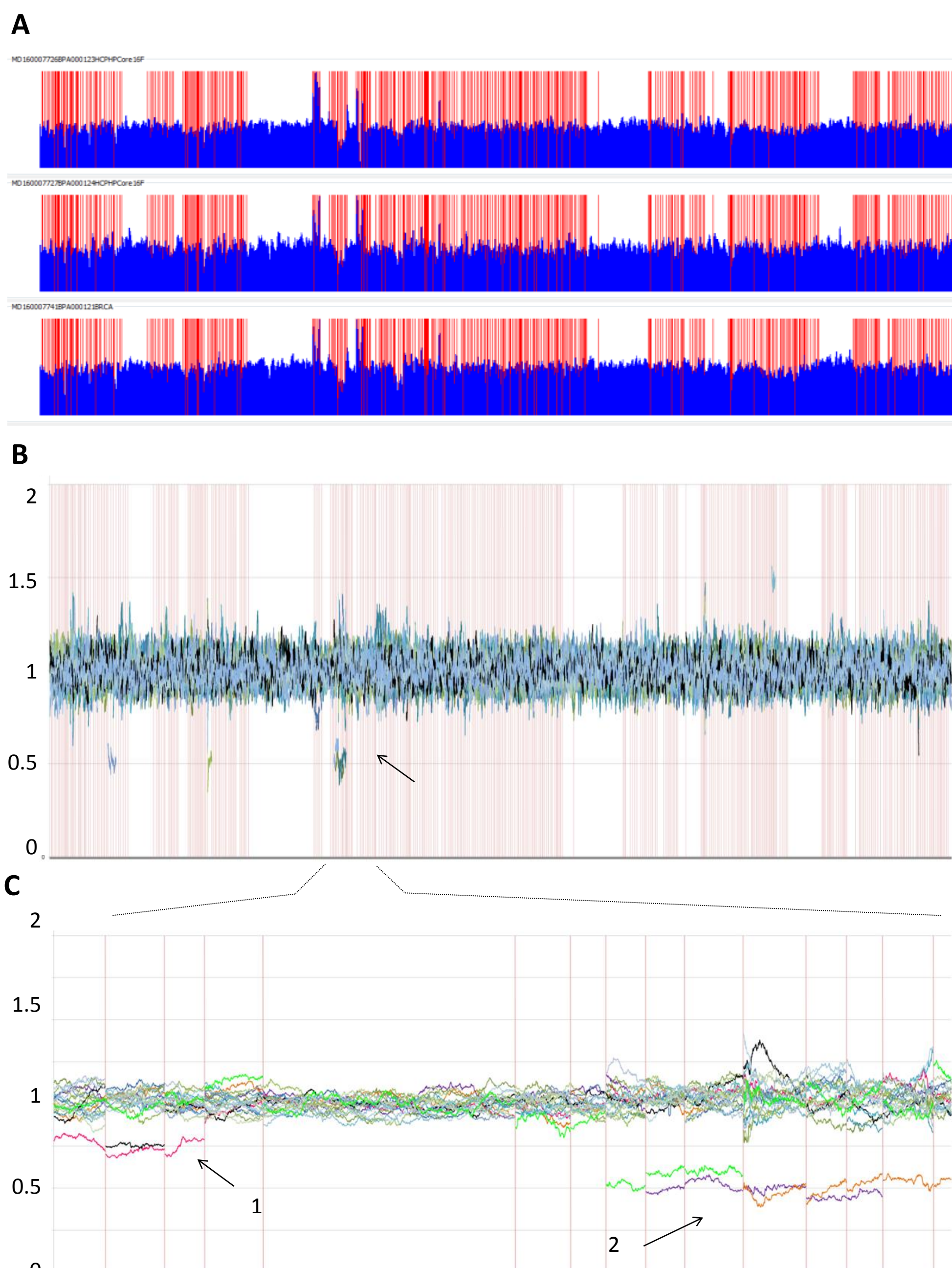

Figure 2: (A) Sequence coverage depth for 3 representative patient screens for CANcer panel (25 genes). (B) Normalized copy number plot demonstrating deletion detection at *PMS2* gene (arrow); (B) Zoomed in at the *PMS2* gene shows three *PMS2* CNVs identified in the 5' region of the gene with a ratio of 0.5 (arrow 2), while other two (arrow 1) were in the region of high homology with *PMS2CL* and were identified by a deletion ratio of 0.75. X-axis indicates gene-exon locations

Results cont.

Detection of Mitochondrial deletion associated with the Kearns-Sayre Syndrome (KSS) with as little as 10% heteroplasmy (Figure 3), homozygous deletions and gene triplications (Figure 4), as well as breakpoint determination (Figure 5)
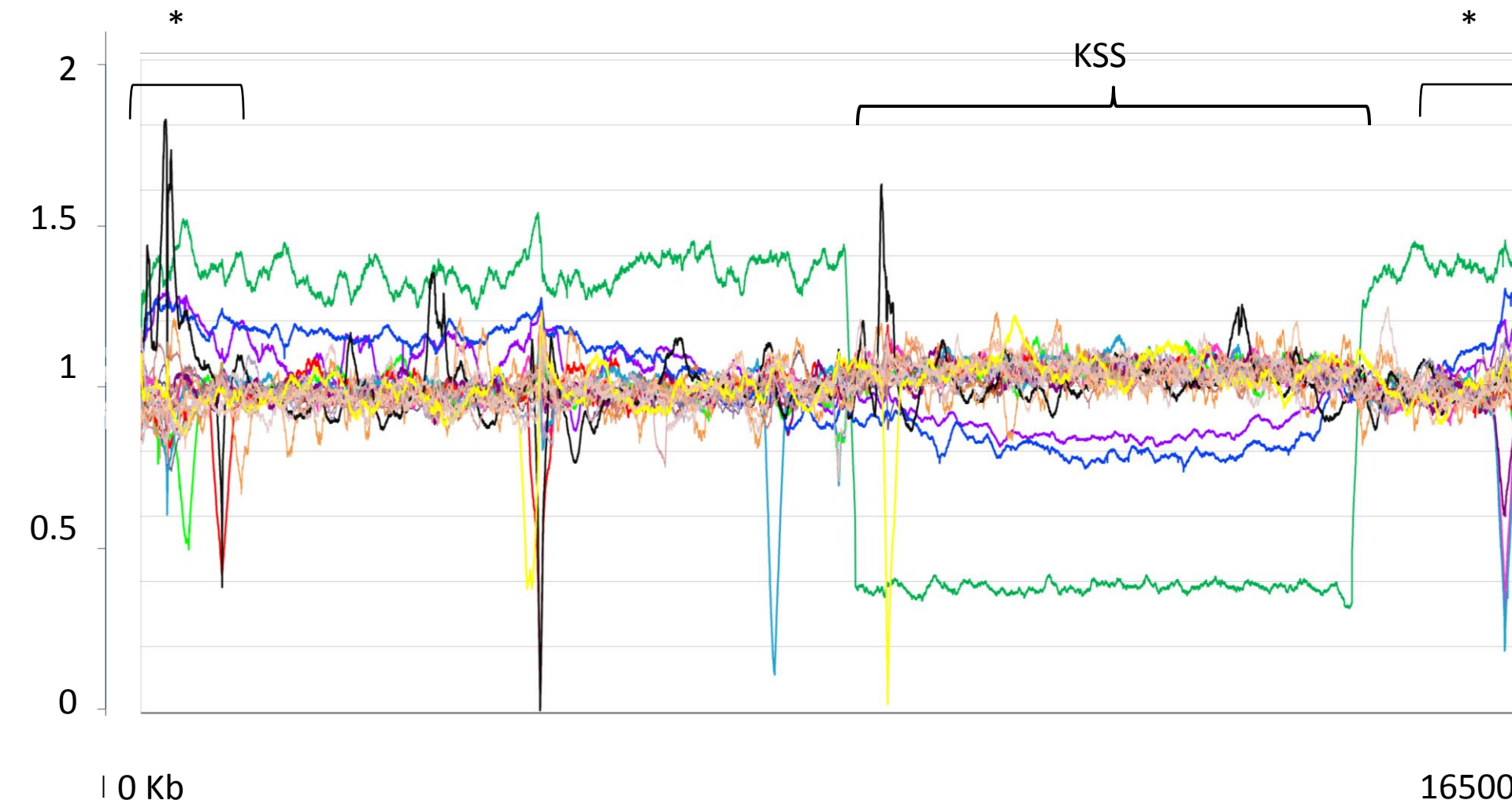

Figure 3: mDNA Panel; Identification of patients with KSS, with approx. 10% heteroplasmy (in blue and pink) and approx. 50% heteroplasmy (in green). * indicates hypervariable D-loop region (m.16,024 to m.576).
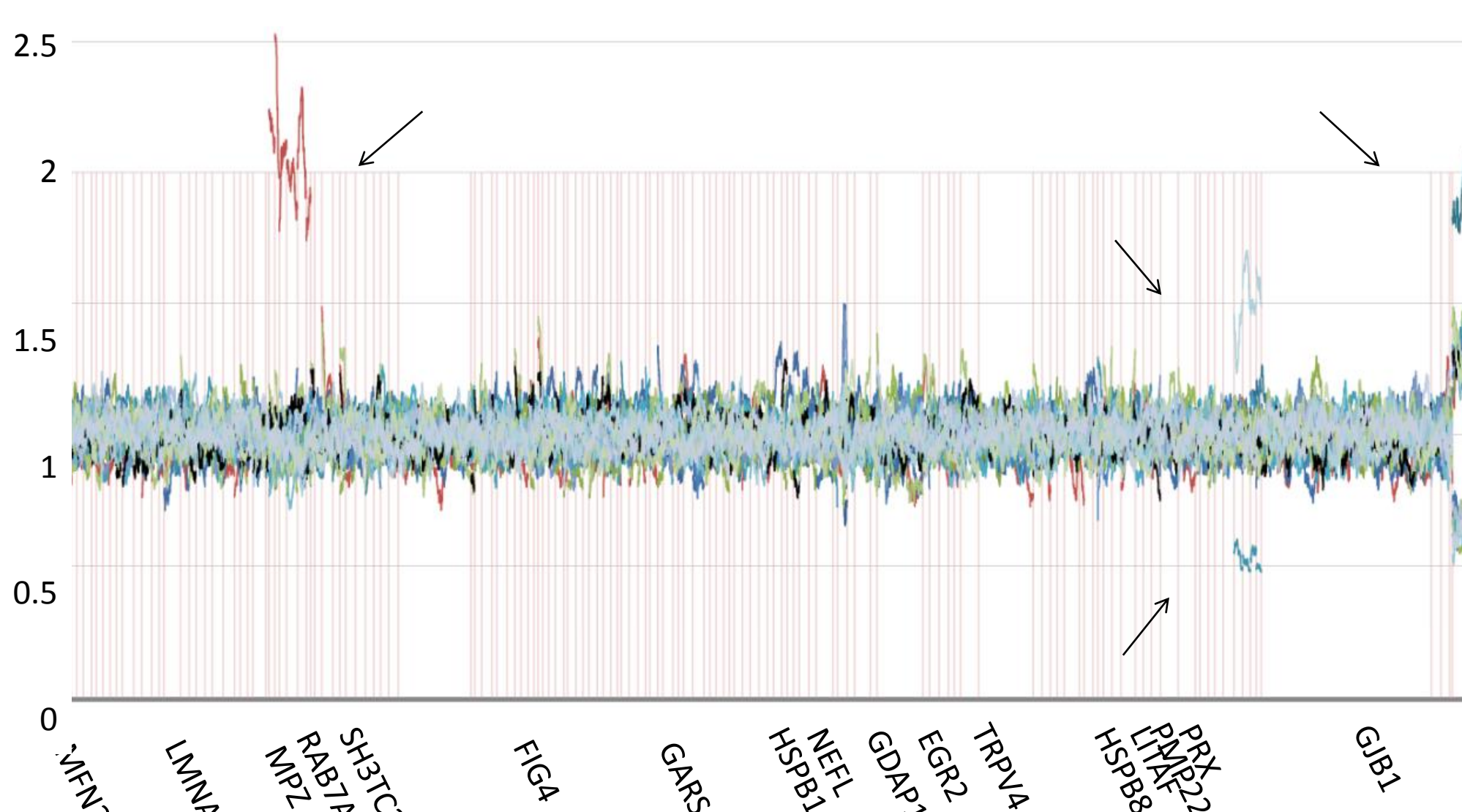

Figure 4: CMT panel; Identification of Trisomy X, deletions and duplications, and a patient with triplication at MPZ gene.
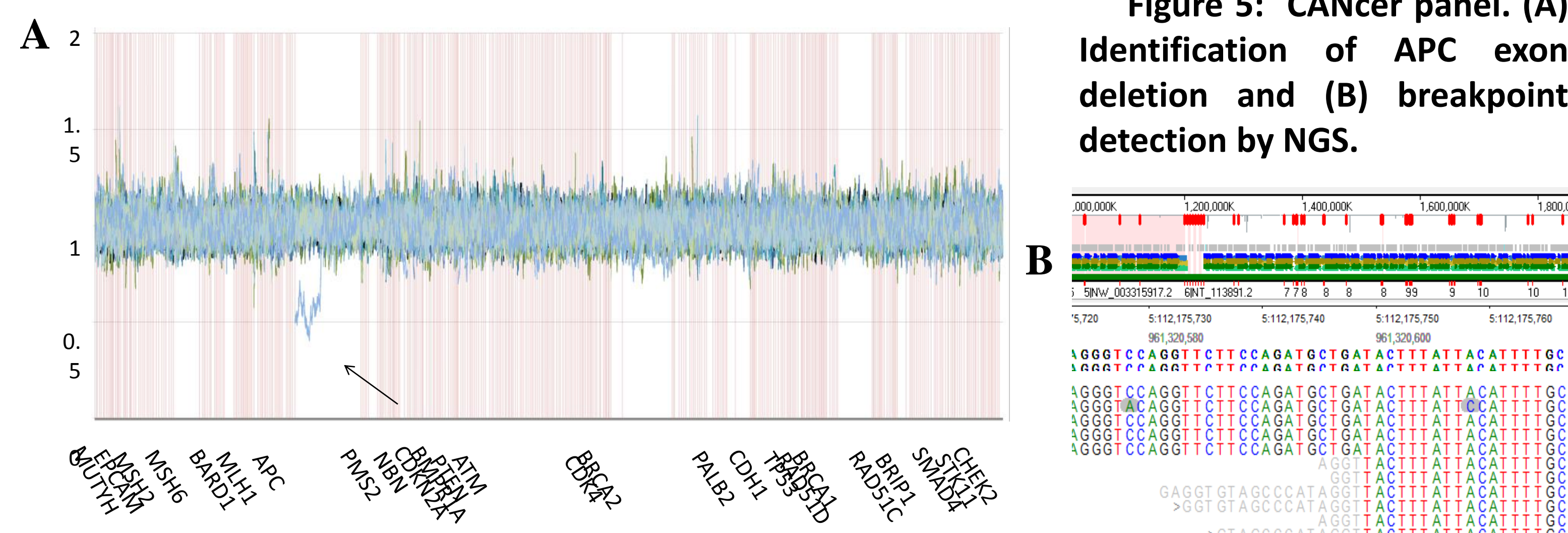

Figure 5: CANcer panel. (A) Identification of APC exon deletion and (B) breakpoint detection by NGS.

Table 1: Summary of CNV identified by NGS and confirmation technique in retrospective and prospective cohorts

	# tests	Detected by MLPA*	Detected by NGS	False negatives	Sensitivity (%)	False positives	Rate of false positives (%)
Retrospective							
Assorted	48	6	6	0	100	-	-
BRCA	120	9	9	0	100	-	-
CANcer	60	10	10	0	100	-	-
CMT	46	6	6	0	100	-	-
Dyslipidemia	24	6	8	0	100	-	-
Epilepsy	0	-	-	-	-	-	-
HypF	0	-	-	-	-	-	-
LSD/UCD	22	2	2	0	100	-	-
Mitochondrial	71	4	4	0	100	-	-
Total	391	43	45	0	100	-	-
Prospective							
Assorted	421	4	7	-	-	3	0.7
BRCA	502	1	4	-	-	3	0.6
CANcer	576	6	17	-	-	11	1.9
CMT	619	88	92	-	-	4	0.6
Dyslipidemia	0	-	-	-	-	-	-
Epilepsy	36	0	0	-	-	-	-
HypF	116	0	3	-	-	3	2.6
LSD/UCD	12	0	0	-	-	-	-
Mitochondrial	93	7	7	-	-	0	0
Total	2375	106	130	-	-	24	1.0

Conclusion

We have validated a clinical-grade NGS pipeline to detect SNV and CNV in a single assay. Our method showed 100 % sensitivity and high specificity for all panels, outperforming the "gold standard" Sanger sequencing and MLPA.

Schenkel LC, Kerkhof J, Stuart A, Reilly J, Eng B, Woodside C, Levstik A, Howlett CJ, Rupar AC, Knoll JH, Ainsworth P, Waye JS, Sadikovic B: Clinical Next-Generation Sequencing Pipeline Outperforms a Combined Approach Using Sanger Sequencing and Multiplex Ligation-Dependent Probe Amplification in Targeted Gene Panel Analysis. J Mol Diagn 2016