The Journal of Molecular Diagnostics, Vol. 17, No. 5, September 2015

the Journal of Molecular Diagnostics
jmd.amjpathol.org

A Systematic Comparison of Traditional and Multigene Panel Testing for Hereditary Breast and Ovarian Cancer Genes in More Than 1000 Patients

Stephen E. Lincoln,* Yuya Kobayashi,* Michael J. Anderson,* Shan Yang,* Andrea J. Desmond,† Meredith A. Mills,‡ Geoffrey B. Nilsen,* Kevin B. Jacobs,* Federico A. Monzon,* Allison W. Kurian,‡ James M. Ford,‡ and Leif W. Ellisen†§

From Invitae, San Francisco, California; the Massachusetts General Hospital Cancer Center,† Boston, Massachusetts; the Stanford University School of Medicine,‡ Stanford, California; and the Harvard Medical School,§ Boston, Massachusetts*

Accepted for publication April 28, 2015.

Address correspondence to Stephen E. Lincoln, B.S., Invitae, 458 Brannan St, San Francisco, CA 94107. E-mail: steve.lincoln@invitae.com.

Gene panels for hereditary breast and ovarian cancer risk assessment are gaining acceptance, even though the clinical utility of these panels is not yet fully defined. Technical questions remain, however, about the performance and clinical interpretation of gene panels in comparison with traditional tests. We tested 1105 individuals using a 29-gene next-generation sequencing panel and observed 100% analytical concordance with traditional and reference data on >750 comparable variants. These 750 variants included technically challenging classes of sequence and copy number variation that together represent a significant fraction (13.4%) of the pathogenic variants observed. For *BRCA1* and *BRCA2*, we also compared variant interpretations in traditional reports to those produced using only non-proprietary resources and following criteria based on recent (2015) guidelines. We observed 99.8% net report concordance, albeit with a slightly higher variant of uncertain significance rate. In 4.5% of BRCA-negative cases, we uncovered pathogenic variants in other genes, which appear clinically relevant. Previously unseen variants requiring interpretation accumulated rapidly, even after 1000 individuals had been tested. We conclude that next-generation sequencing panel testing can provide results highly comparable to traditional testing and can uncover potentially actionable findings that may be otherwise missed. Challenges remain for the broad adoption of panel tests, some of which will be addressed by the accumulation of large public databases of annotated clinical variants. *(J Mol Diagn 2015, 17: 533—544; http://dx.doi.org/10.1016/j.jmoldx.2015.04.009)*

Multigene panel testing has proved useful as a diagnostic tool for disorders where similar phenotypes can be influenced by multiple genes.[1] Recent advances in next-generation DNA sequencing technology (NGS) have enabled these clinical tests and made them increasingly inexpensive to perform.[2,3] For hereditary cancer syndromes, studies have shown that NGS-based panel tests can uncover potentially actionable findings that may be missed by traditional testing paradigms.[4—12] Validation studies of clinical NGS assays for hereditary cancer genes have correspondingly been published,[4,7,11,13,14] and certain guidelines exist for their clinical implementation.[15—18] Patient management experience using these hereditary cancer panels is growing,[4,19,20]

Supported by The Friends Fighting Breast Cancer (L.W.E.), the Tracey Davis Memorial Fund (L.W.E.), and the Breast Cancer Research Foundation (A.W.K. and J.M.F.).

Disclosures: S.E.L., Y.K., M.J.A., S.Y., G.B.N., and F.A.M. are employees of Invitae and own stock and/or stock options. J.M.F. is a paid member of Invitae's advisory board. Separately from this study, J.M.F. and A.W.K. receive research funding from Myriad Genetics. L.W.E. is a consultant to Bioreference/GeneDx Laboratories. S.E.L. owns stock in Illumina, whose instruments were used in this study. The funding organizations had no role in the design, conduct, or reporting of this study. Invitae provided the 29-gene panel test results used in this study. This study was an academic collaboration and not a sponsored research project: no other funding or compensation was provided by Invitae.

This paper conforms to the STARD guidelines (*http://www.stard-statement.org*) for reporting of diagnostic cohort studies.

Current address of K.B.J., 23andMe, Inc., Mountain View, CA.

http://dx.doi.org/10.1016/j.jmoldx.2015.04.009

Table 1 Study Population

Population	Group	No. of patients	Description	Previous testing
Clinical cases (n = 1062)	Clinical referral	735	Patients prospectively accrued following NCCN guidelines for HBOC	Traditional clinical testing for *BRCA1* and/or *BRCA2* in most cases, with occasional testing for other genes
	History enriched (n = 327)	209	Retrospective cases from a clinical biobank containing particularly high-risk patients	
		118	Cases referred because of a known pathogenic variant in family	Clinical single-site testing
Reference samples (n = 43)	Positive reference samples	36	Reference samples selected from public biobanks	Samples carry known pathogenic variants in specific genes
	Genome reference samples	7	Reference samples from public biobanks with high-quality WGS data	Variants in 29 cancer genes extracted from WGS data
Total		1105		

Individuals included in this study, with their selection criteria. Previous test results for *BRCA1* and/or *BRCA2* were available for 92% (n = 975) of the 1062 clinical cases. All of the reference samples had previous test data. The specific type and scope of testing varied (Supplemental Table S2).

HBOC, hereditary breast/ovarian cancer; NCCN, National Comprehensive Cancer Network; WGS, whole genome sequencing.

although the clinical utility of these panels is not yet fully established[21,22] and the appropriate routes for clinical deployment of such tests remain under discussion.[23]

Several technical questions also remain about these new tests. NGS has traditionally had analytical limitations[24] compared with established technologies, such as Sanger sequencing,[25] quantitative PCR,[26] multiplex ligation-dependent probe amplification (MLPA),[27] and copy number microarrays.[28] If panel tests are to replace traditional single-gene tests in appropriate situations, further evidence is required to show that NGS can meet the analytic performance standards of these established methods, particularly on those classes of variants that are known to be most challenging for NGS. In addition, questions have long been raised about the potential for inconsistent variant interpretations between laboratories because of limited access to proprietary data and because of differences in interpretation criteria.[29,30] This is an increasingly relevant area for study, because NGS-based tests from multiple laboratories have emerged in recent years[31] and new guidelines for the interpretation of sequence variants (ISV) have also emerged.[32]

To help address these technical questions, particularly as they apply to hereditary breast/ovarian cancer (HBOC), we tested 1062 patients with an NGS-based 29-gene hereditary cancer panel. These individuals were indicated for HBOC risk assessment under National Comprehensive Cancer Network (NCCN) guidelines,[33] and most had previously received clinical testing for *BRCA1* and/or *BRCA2* from another laboratory. We supplemented these data with additional confirmatory testing and with data from 43 additional reference samples to evaluate NGS performance over more genes and variants. We classified variants following a system based on the recent ISV guidelines, and we used only broadly available, non-proprietary resources to do this. In total, these data allowed us to compare both the analytical and clinical interpretation results from traditional BRCA testing with an NGS-based gene panel.

Materials and Methods

Patients, Samples, and Previous Test Data

Samples were compiled from multiple sources (Table 1 and Supplemental Table S1), and most had previous genetic data available for comparison (Supplemental Table S2).

Seven-hundred thirty-five patients referred for HBOC counseling and/or testing under NCCN guidelines[33] were prospectively recruited at two academic medical centers: the Stanford Clinical Cancer Genetics Program (Stanford, CA; 2002-2012) and the Massachusetts General Hospital Center for Cancer Risk Assessment (Boston, MA; 2013-2014). A further 118 patients referred to either center because of known familial mutations were recruited but considered separately in this analysis. An additional group of 209 patients was recruited at Massachusetts General Hospital Center for Cancer Risk Assessment (2000-2012) on the basis of high-risk personal and family features, but not under uniform criteria, and they are also considered separately. Of the total 1062 patients, 975 (92%) had previously received traditional *BRCA1* and/or *BRCA2* tests from Myriad Genetics (Salt Lake City, UT), and a small subset (4%) had undergone tests for other genes or multigene panels, as had been clinically indicated. A subset of these patients (n = 175) had been analyzed in our prior work using a research panel,[4] although insufficient material remained to retest all 198 patients from that previous study here.

Thirty-six reference samples carrying known pathogenic variants were selected from two public biobanks: the Coriell Institute (Camden, NJ) and the National Institute for Biological Standards and Control (Hertfordshire, UK) (Supplemental Table S3).

Table 2 List of 29 Genes Tested by Subpanel

Subpanel name	Genes	Total	Gene names
BRCA1/2	2	2	*BRCA1*, *BRCA2*
Other high-risk breast/ovarian	4	6	*CDH1*, *PTEN*, *STK11*, *TP53*
Moderate-risk breast/ovarian	6	12	*ATM*, *BRIP1*, *CHEK2*, *NBN*, *PALB2*, *RAD51C*
Lynch syndrome	5	17	*EPCAM*, *MLH1*, *MSH2*, *MSH6*, *PMS2*
Other hereditary cancer syndromes	11	28	*APC*, *BMPR1A*, *SMAD4*, *CDK4*, *CDKN2A*, *PALLD*, *MET*, *MEN1*, *RET*, *PTCH1*, *VHL*
MUTYH	1	29	*MUTYH*

Seven additional reference samples with publicly available whole genome sequence (WGS) data were selected (Supplemental Figure S1). One was NA12878, the pilot reference standard developed by the Genome in a Bottle consortium (National Institute for Standards and Technology, Gaithersburg, MD; *http://www.genomeinabottle.org*, last accessed February 19, 2015).[34] All seven came from fully sequenced pedigrees and had data from multiple WGS platforms. Thus, high-accuracy reference calls could be constructed by both integrating the different WGS data sets and using mendelian analysis with phasing.[34–36]

Institutional Review Board Approvals

All patients signed informed consents approved by the Institutional Review Boards of Stanford University (Stanford, CA) or the Dana-Farber Harvard Cancer Center (Boston, MA). Reference samples were used under the terms of their material transfer agreements.

Gene Panel

Twenty-nine genes (Table 2 and Supplemental Table S4) were selected on the basis of their documented role in cancer risk[37–68] and for technical feasibility using the methods described below. These genes were included in a larger NGS assay of 220 genes, most of which are not related to hereditary cancer. We limited our analysis to these 29 genes in this study. Of the 29 genes, 28 are autosomal dominant or have both dominant and recessive cancer phenotypes of varying penetrance. *MUTYH* was considered separately as a recessive gene, although there is some evidence of elevated cancer risk in monoallelic carriers.[69,70] The specific version of the assay used in this study had known limitations and did not include exons 12 to 15 of *PMS2*, because of the known pseudogene (*PMS2CL*), or regions of *CHEK2* other than the c.1100 delC position, because of multiple homologous genomic loci (including the *CHEK2P2* pseudogene).

Next Generation Sequencing

Genomic DNA was extracted from whole blood using a QiaSymphony (Qiagen, Hilden, Germany). Targeted genes were captured using Agilent (Santa Clara, CA) SureSelect probes or Integrated DNA Technologies (Coral, IL) xGen Lockdown probes at positions where SureSelect yield was inadequate. NGS[71] was performed on the Illumina (San Diego, CA) MiSeq or HiSeq 2500 to at least 450× average coverage of 2 × 150 reads, with a minimum of 50× required at every targeted position. Stringent process controls were used to minimize read-depth variability, and up to eight anonymous blood samples were used as control specimens in each run to measure remaining coverage variability.

Bioinformatics

Reads were aligned to the reference human genome sequence GRCh37[72] using Novoalign (Novocraft Technologies, Selangor, Malaysia). Sequence variants were called using a collection of algorithms, including the Genome Analysis Toolkit Unified Genotyper,[73] Freebayes,[74] and Coalgen (unpublished data). Copy number variants (CNVs; alias del/dup events) were called using read-depth analysis by CNVitae.[75] CNV analysis was not performed on *PALLD* or *MET* in this specific assay owing to less predictable read-depth variability. Of 1062 clinical samples, 79 predated the production implementation of CNVitae and, thus, had no NGS CNV assay performed (Supplemental Table S5). Split-read analysis[76] was performed in a manner similar to that described elsewhere,[77] although we used this technique at all loci rather than just CNV edges. Split-read analysis allowed us to detect certain structural variants that can evade both read-depth analysis (by having a breakpoint within a small assay target) and indel detection (by being too large or by having a breakpoint outside of the targeted regions).

Quality Control

Quality control (QC) parameters included read qualities, mapping qualities, depth of coverage, PCR duplication rates, on/off-target rates, insert size distribution, allelic balance, uniformity of coverage versus controls, and measures of sample contamination (Supplemental Table S6). More than 99% of samples produced data that passed our QC criteria without the need for gap-filling assays, such as Sanger sequencing (Supplemental Table S5). Approximately 10% to 15% of samples required multiple runs to meet these criteria, which we usually performed by repeating both DNA

extraction and NGS. Most of these reruns were only required to pass coverage variability metrics related to CNV calling: criteria that apply to the calling of sequence variants were often (>95% samples) met on the first attempt. We found that this rerun rate decreased over time as the technologists gained greater experience with the protocols and as further automation was used in their execution.

Variant Interpretation

Updated ISV guidelines were recently established by the American College of Medical Genetics and Genomics and the Association of Molecular Pathology. We used a point-based system based on the last draft of these ISV guidelines.[78] That system remains consistent with the final version of these guidelines.[32] We classified variants using the five-class system as pathogenic (P), likely pathogenic (LP), variant of uncertain significance (VUS), likely benign (LB), or benign (B). Only non-proprietary data sources were used for this classification, including the published scientific literature, the Universal Mutation Database,[79] and the International Society of Gastrointestinal Hereditary Tumours database.[80] Population databases, such as the 1000 Genomes Project (*http://www.1000genomes.org*, last accessed November 1, 2014)[81] and the Exome Variant Server (National Heart, Lung, and Blood Institute GO Exome Sequencing Project, Seattle, WA; *http://evs.gs.washington.edu/EVS*; last accessed November 1, 2014), were used for allele frequencies.

The previous clinical tests used proprietary data[30] and somewhat different interpretation criteria.[82] They also used different nomenclature (eg, deleterious and favor polymorphism), which we mapped onto the ISV five-class nomenclature.

Orthogonal Confirmation

Variants were tested using Sanger sequencing[25] or MLPA.[27]

Blinding

NGS sequencing, production bioinformatics, data QC, and clinical interpretation were performed blind to the previous test results. Raw (fastq) data for older specimens in this study were reprocessed after unblinding to produce a consistent data set with uniform bioinformatics and QC (Supplemental Table S5). Interpretations for some variants were updated over the course of this study following an objective process (as is used in clinical practice to amend reports) based solely on the appearance of new evidence or criteria. Laboratory staff with access to the previous test data after unblinding were not involved in this reprocessing or interpretation updates. Newer specimens without reprocessing ($n = 313$) showed the same degree of concordance as older specimens, suggesting that no bias was introduced.

Statistical Analysis

Binomial proportion CIs were calculated using the Jeffreys method. We considered the possible overestimation of performance from discrepant analysis,[83] but given that all discordances turned out to be cases where corresponding tests had not been performed ($n = 143$) or where the reference data were clearly incorrect ($n = 3$), it was determined to not be a significant concern in this study.

Data Submission

De-identified variants and their interpretations from this study have been submitted to ClinVar (National Center for Biotechnology Information, Bethesda, MD; *http://www.ncbi.nlm.nih.gov/clinvar*, last accessed November 1, 2014).[84] Accession numbers are available in Supplemental Table S7.

Results

Spectrum of Variation

From the 29-gene panel, 58,708 variants were observed across our 1105 individuals. The number of variants in each gene varied considerably and did not strongly correlate with gene size (Supplemental Table S8). The vast majority of these (>99.5%) were single-nucleotide variants, and most (>90%) were common polymorphisms present in the 1000 Genomes Project data set[81] at a minor allele frequency of 1% or greater.

Pathogenic Variants

In 1062 clinical cases, 260 variants classified as P or LP (collectively P/LP) were observed (Figure 1 and Supplemental Table S9). Focusing on the referral cohort ($n = 735$) as our most clinically representative population, P/LP variants in *BRCA1* or *BRCA2* (collectively, BRCA) were observed in 9.0% ($n = 66$) patients, as expected given our criteria for accrual.[85] Among the BRCA-negative patients, 3.9% ($n = 26$) carried P/LP variants in other dominant-acting genes. Almost all of these non-BRCA findings were in genes with a known association to breast/ovarian cancer, or were in genes associated with Lynch syndrome. The only exception in the referral cohort was a single patient with a *CDKN2A* pathogenic variant. The most common non-BRCA findings were in *ATM* (five cases), *PALB2* (five cases), *CHEK2* (three cases), and the Lynch syndrome genes (*MLH1*, *MSH2*, *MSH6*, and *PMS2*; collectively, eight cases). In addition, 2.7% of patients were monoallelic carriers of a P/LP variant in *MUTYH*, although we saw no biallelic *MUTYH* carriers (ie, homozygotes or compound heterozygotes). In the history-enriched group, a much larger fraction (40%) of patients were positive for *BRCA1* or *BRCA2*, as expected, and more of the BRCA-negative individuals (6.1%) were positive for other dominant-acting genes.

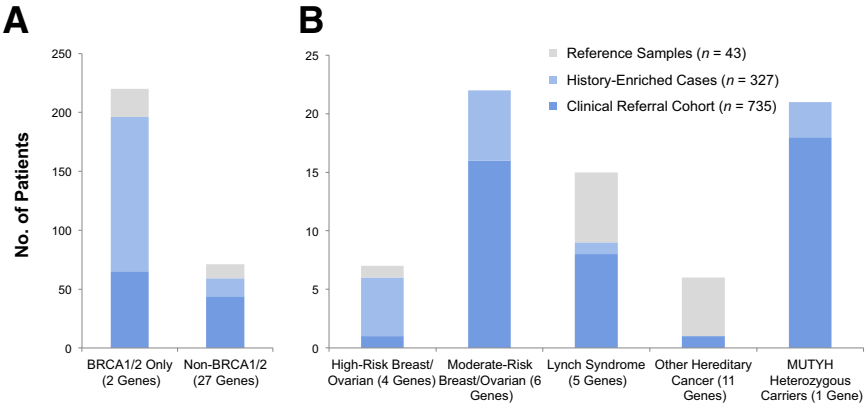

Figure 1 Pathogenic variants by subpanel. Number of cases with pathogenic or likely pathogenic variants observed in *BRCA1/BRCA2* and in 27 other genes (**A**) and in these 27 other genes by subpanel (**B**). The clinical referral cohort represents our best estimate of prevalence in a clinically representative population.

A small fraction (4%) of the patients had previously received one or more tests for non-BRCA genes (Supplemental Table S2) in addition to ($n = 30$) or instead of ($n = 13$) tests for BRCA. These test results were usually negative. Only 3 of 26 non-BRCA P/LP variants in the referral cohort were detected by any previous test: two (in *CHEK2* and *CDH1,* respectively) by a gastrointestinal cancer panel (ColoNext; Ambry Genetics, Aliso Viejo, CA) and one (in *PALB2*) by a single-gene test. In this study, most non-BRCA findings were detected only through uniform application of the 29-gene panel.

Some unexpected results were observed among the 118 individuals who had previously received only single-site testing for familial pathogenic variants in *BRCA1* or *BRCA2*. Two, negative for those familial variants, had pathogenic variants detected by the panel in different genes (*ATM* and *MSH2*, respectively). These were confirmed and determined unlikely to be the result of sample swaps or clinical record errors; however, we were unable to recontact the two patients to reconfirm these findings in independent specimens. A third individual carried a familial *BRCA2* pathogenic variant in addition to a previously undetected pathogenic variant in *ATM*, whereas a fourth carried a familial *BRCA2* pathogenic variant and a previously undetected *BRCA1* pathogenic variant.

Variants of Uncertain Significance

Among the 1062 clinical cases, 41.0% had at least one VUS among the 29 genes, with 11.4% having two or more VUSs (Figure 2). As expected, the prevalence of VUSs increases considerably with the number of genes tested, and indeed it increases significantly faster than the additional yield of P/LP variants (Figure 1). Most (68%) of these VUSs were rare missense variants not present in the 1000Genomes Project database.[79]

Technically Challenging Variants for NGS

A small minority (<0.1%) of the 58,708 variants observed were of types known to be relatively challenging for NGS, defined herein as either large indels (sequence deletions >10 bp or insertions >5 bp); CNVs (alias del/dup events), including gains and losses as small as one exon; block substitutions (delins events); short-range haplotypes (variants within 25 bp); or known homopolymer-associated variants. Given the analytic results described below, we believe this low rate reflects the prevalence of these types of variants more than assay sensitivity limitations.

In contrast, a strikingly disproportionate fraction (13.4%) of the 260 P/LP variants in the clinical populations were of these challenging types (Figure 3). CNVs were 8.4%, almost half of which (3.8% of the P/LP total) affected only a single exon, consistent with findings reported elsewhere using microarrays.[86,87] Many (15 of 29) of the CNVs were either rare or novel, highlighting the value of unbiased methods to detect these variants, as opposed to limited genotyping assays. The largest sequence-level variant we observed (*BRCA2*: c.9203del126) was detected only by split-read analysis (Supplemental Figure S2) and not by copy number (CNVitae) or standard indel callers (Genome Analysis Toolkit and Freebayes). The largest sequence insertion we observed was a third copy of a

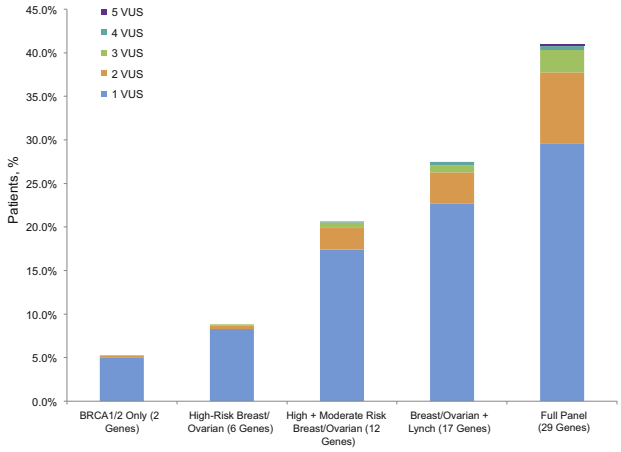

Figure 2 Prevalence of variants of unknown significance (VUSs). Cumulative fraction of clinical cases with one or more VUSs reported, irrespective of pathogenic variants observed, as the scope of testing increases.

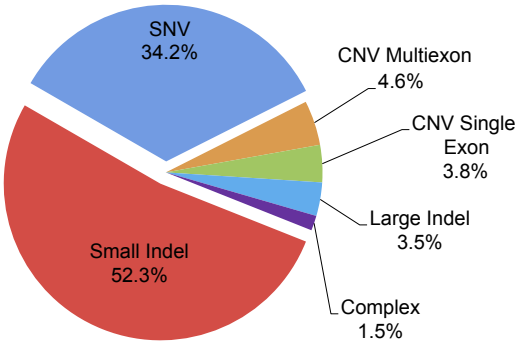

Figure 3 Pathogenic variants by type. Pathogenic variants ($n = 260$) among the clinical cases ($n = 1062$) by variant type. A significant fraction (13.4%) of the pathogenic variants are of types known to be technically challenging for next-generation sequencing, even though they are a small fraction (<0.1%) of the total set of variants. Single-nucleotide variant (SNV) and copy number variant (CNV) are shown.

GC-rich 24-bp tandem duplication (*CDKN2A*: c.9_32dup24), which produced a complex, yet strong, signal by NGS (Supplemental Figure S3).

Analytic Validation

We approached analytic validation by first comparing all results from the previous testing with the 29-gene panel data (Supplemental Figure S4). Any concordant variant was considered a confirmed true positive for the panel, otherwise a false negative (FN). Given limitations in the previous testing, however (Supplemental Table S2), we knew that the reverse comparison would not be as simple: many panel test results would not be present in the previous reports, making it harder to measure false positives. Furthermore, because the previous reports usually omitted variants classified as B or LB, analytic concordance could be confounded with interpretation concordance. We addressed these issues by also orthogonally confirming 143 previously unreported variants, prioritizing those that were P or LP, were technically important (ie, any other indels and CNVs), or helped answer a specific question, in particular whether VUS versus negative report differences were a result of an interpretation difference or an analytic difference. We examined the previous test records and found that all 143 were not previously reported because either a corresponding test had not been previously performed or they were likely filtered from the previous clinical test results by virtue of being classified as benign. Thus, we have no evidence of FNs in the previous test results.

In total, 750 variants were considered where the 29-gene panel could be directly compared with gold standard data, from either the previous testing or orthogonal confirmation. All 750 were determined to be concordant true positives (Table 3); no false positives or FNs were observed in the 29-gene panel results. More important, these 750 represented a diversity of variant types (Table 4), including 48 of the technically challenging classes listed above (eg, CNVs and large indels). Of these 48, 10 were not previously reported, again because corresponding tests were not previously performed. The 750 also represented a diversity of classifications: 38% were P/LP, 13% VUS, and 49% B/LB, with most of the benign variants contributed by the seven reference genome data sets. Approximately half (372 of 750) of these variants were in genes other than *BRCA1* or *BRCA2*, and all 29 genes except one (*CDK4*) had at least one variant in this set of 750 (Supplemental Table S8). Most (607 of 750) of these variants were reported by the previous tests, providing the most unbiased view of sensitivity for the NGS panel (607 of 607 = 100%; 95% CI, 100%–99.59%).

To estimate the true-negative rate, we counted regions with concordant negative results, disregarding benign variants and counting only those cases for which the 29-gene panel and the previous tests were complete (ie, full-gene sequencing or full copy number assessment was performed). This allowed us to calculate CIs for specificity on a per–base-pair or per-exon basis (Table 3). Calculated instead on a per-test basis, counting each comparable gene by individual combination as one test, the CI for specificity and true-negative count is 100% to 99.86% ($n = 1839$) for sequence variants and 100% to 99.76% ($n = 1026$) for CNVs.

Table 3 Analytic Concordance

	Previous testing or independent confirmation		
29-Gene NGS panel	Variant present	Variant not present	Result
Variant detected	750 True positives	0 False positives	100% Sensitivity (95% CI for sequence, 100%–99.7%) (95% CI for CNV, 100%–91.8%)
Variant not detected	0 False negatives	Sequence, 15.0 M true negative Base pairs CNV, 22.2 K true negative exons	100% Specificity (95% CI for Sequence, 100%–99.99998%) (95% CI for CNV, 100%–99.989%)

Concordance of variant calls in all samples ($n = 1105$) between the 29-gene panel and the independent test results. Variants are counted regardless of pathogenicity if a corresponding test was performed both previously and by the 29-gene panel, or an independent confirmation experiment was performed. The CIs for sensitivity are computed on a per-variant basis; and for specificity, computed on a per–base-pair (sequence variants) or per-exon (CNV) basis.

CNV, copy number variant; K, thousand; M, million.

Table 4 Variants in Analytic Study

	Type	Variants	Sizes or genes
Sequence	SNVs	549	
	Sequence deletions <10 bp	125	
	Sequence deletions ≥10 bp	9	126, 40, 19, 15, 11 bp
	Sequence insertions <5 bp	31	
	Sequence insertions ≥5 bp	4	24, 5 bp
	Complex variants	6	Delins, haplotypes, homopolymer associated
Copy number	Single-exon deletions	9	*BRCA1*, *BRCA2*, *MSH2*, *PMS2*
	Deletions of multiple exons or whole gene	10	*BRCA1*, *MSH2*, *RAD51C*
	Single-exon duplications	4	*BRCA1*, *MLH1*
	Duplications of multiple exons or whole gene	6	*BRCA1*, *BRCA2*, *NBN*, *SMAD4*
	Total	750	

Variants in all samples ($n = 1105$) selected for the analytic validation study. This list includes both variants detected previously and those detected by the 29-gene panel and then subjected to orthogonal confirmation (Supplemental Figure S4). All 750 were confirmed as true positives (Table 3).

SNV, single-nucleotide variant.

BRCA Interpretations

Among the 1062 clinical cases, 975 had previous *BRCA1* and/or *BRCA2* tests that could be compared with the 29-gene panel test results. To determine how often differences in variant interpretation could lead to significantly different test reports, we first categorized patients as BRCA positive (P or LP variant in *BRCA1* or *BRCA2*), uncertain (no P/LP variants but at least one VUS), or BRCA negative (no P/LP variants nor VUSs). Furthermore, because clinicians manage patients with uncertain reports according to personal and family history, and not their genetic test results,[33] we compared positive with not positive (ie, uncertain or negative) reports (Table 5). On this per-patient basis, we see 99.8% concordance (973 of 975; 95% CI, 99.3%–99.9%) between the previous reports and NGS panel test results (Table 5). The two discordant patients had variants reported by the previous laboratory as pathogenic (*BRCA1*:c.4986+3G>C and *BRCA2*:c.316+5G>A, respectively), for which there is inadequate publicly available evidence to support pathogenicity (Supplemental Table S10).

We further examined specific interpretations for the 1847 comparable BRCA variant observations in these 975 patients. The vast majority (1765 of 1847) had exactly concordant interpretations in the five-class system (Supplemental Table S11), despite the differences in criteria and evidence used. Excluding the two variants mentioned above, the remaining differences had less substantial clinical significance: most differences (65 of 80) were in variants classified as LB by one laboratory and classified as B or entirely omitted from reports by the other. Five were differences between P and LP, and the remaining 10 were differences between VUS and B or LB (Supplemental Table S10). Many of these differences could result from either having different evidence available or differences in the specific details of interpretation criteria. Overall, 95.6% of 1847 had exactly concordant interpretations, and 99.5% were either exactly concordant or one step apart in the five-class system.

Table 5 Interpretation Concordance for *BRCA1/BRCA2*

BRCA1/BRCA2 results from 29-gene panel	Previous *BRCA1/BRCA2* testing results			
	Positive	Uncertain	Negative	Total
Positive	**188**	0	0	188 (19.3%)
Uncertain	*2*	**30**	*8*	787
Negative	0	*1*	**746**	(80.7%)
Total	190 (19.5%)	785 (80.5%)		975

Concordance of net results for BRCA testing of each patient whenever corresponding tests for *BRCA1* or *BRCA2* were performed ($n = 975$ of 1062 patients). Bold numbers are concordant results, and italicized numbers are discordant results. Of 975 cases, 973 (99.8%) are concordant for positive versus nonpositive results. The positive category includes pathogenic and likely pathogenic variants as well as positive single-site and mutation panel tests. The negative category includes patients with only benign or likely benign variants, or with no variants reported. Considering only tests that were capable of reporting variants of uncertain significance, 4.0% (31 of 769) had uncertain reports from the previous testing.

Discussion

In this study, we compared traditional genetic testing with NGS-based panel testing of 29 hereditary cancer genes in a large population. Most of these individuals had previously received traditional *BRCA* testing, whereas some had other genetic tests or high-quality reference data available. In this comparison, we were able to consider issues of analytical performance, variant interpretation concordance, and yield of various classes of findings from panel testing.

In comparing our NGS panel with the previous data, we observed 100% analytical concordance whenever corresponding tests were performed. Although these results are encouraging for the field, they apply only to the specific gene panel, NGS laboratory protocols, and bioinformatics methods we used. Indeed, some NGS studies have reported much lower specificities[13] and/or sensitivities.[88] Some studies report good NGS sensitivity but include few, if any, of the most challenging variant types.[13,14] These

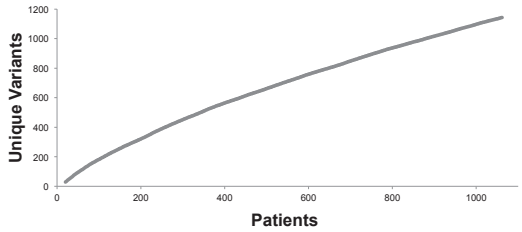

Figure 4 Accumulation of new variants requiring interpretation. Accumulation of new variants in 29 genes requiring clinical interpretation as patients are added ($n = 1062$). The slope at the end is 0.7 new variants per patient. Variants with high minor-allele frequencies (>3% in the 1000 Genomes Project data) are automatically classified as benign and are not included in this analysis. Data shown are a smoothed average over 10 randomized orders of patients.

comparative results highlight the importance of carefully selecting and validating specific methods for each NGS-based test.

In contrast with the methods used in this study, some laboratories complement NGS with microarrays for CNV analysis.[13,87] Our NGS-based CNV detection was highly accurate, even though many of the CNVs present were small (single exons). Clearly, however, comprehensive testing can require a mix of technologies. Two challenging regions (exons 12 to 15 of *PMS2* and most of *CHEK2*) were excluded from this study because of pseudogenes. Methods for addressing such loci by NGS are available[89] but were not implemented in our laboratory in time for this study. In the future, NGS may become a universal assay, but at present, comprehensive testing can require specialized NGS protocols or non-NGS techniques. Laboratories must carefully consider which technologies to implement and must fully understand and disclose limitations of each test they offer.

We designed the analytic validation portion of this study to include a broad set of genes and variant types. Despite the many individuals we studied, the total number of challenging variants was still small ($n = 48$), owing to their relative scarcity. Reference samples with accurate WGSs proved useful in assessing our NGS panel across many genes, although unsurprisingly,[34,90] only relatively easy variants (single-nucleotide variants and small indels, none of which were pathogenic) were seen in these otherwise unselected individuals. Furthermore, the WGS data have limitations (including coverage gaps, no-calls, and unassayed variant types)[34] that can render these data less useful for some validation purposes. Reference samples with challenging variants in coding regions of multiple clinically relevant genes would be helpful for future validation studies.

Most laboratories (including ours) orthogonally confirm NGS findings to reduce the risk of false positives, consistent with established guidelines.[16] The high concordance of our data raises questions about the value of confirmation in some cases, as others have also recently discussed.[91] The 2.2014 revision to the NCCN guidelines[92] also expresses concern about potential for NGS FNs, which can be harder to study. We observed no FNs within our panel's analytic range in >1105 individuals, although we potentially could have observed analytic FNs if more thorough previous tests had been performed and had uncovered variants missed by our NGS panel. Assay range limitations seem a more likely source of clinical FNs. With the rapid pace of technology and scientific understanding, there is not always detailed consensus on the specific set of genes, reference transcripts, genomic regions, or variant types that should, at a minimum, be assayed for many clinical applications.

Most of the previous test results in this study came from a single laboratory that used a large proprietary database to aid in variant interpretation.[30,31,82] Efforts to generate open and peer-reviewed databases, such as ClinVar,[84] may help improve quality and consistency between laboratories. The revised ISV guidelines may also help, although a critical element of medical and scientific judgment will continue to play a vital role in variant interpretation. The 99.8% case-level concordance we observed for *BRCA1* and *BRCA2* suggests that the ISV guidelines are robust and generally consistent with established practices, and broadly available data are sufficient to correctly interpret most BRCA variants. Nevertheless, the few discordant cases we observed remain vexing, because we cannot scrutinize the underlying evidence of pathogenicity used by the previous laboratory.

Given such concerns, professional societies, including the American Medical Association, have adopted positions in favor of genetic data sharing (American Medical Association press release, June 18, 2013; *http://www.ama-assn.org/ama/pub/news/news/2013/2013-06-18-new-ama-policies-annual-meeting.page*). Consortia have formed to facilitate these data-sharing efforts, including ClinGen (*http://www.iccg.org/about-the-iccg/clingen*), the Global Alliance for Genomics and Health (*http://genomicsandhealth.org*), and FreeTheData (*http://free-the-data.org*, all accessed November 1, 2014). We believe it is critical that clinical variants and evidence used in clinical decision making be made available for peer review. All of the variants in this study were thus submitted to ClinVar before this manuscript submission.

Data sharing will be particularly important to reduce the number of patients who receive uncertain reports from panel tests, particularly because the prevalence of VUSs increases with the number of genes tested. Genetic counseling practices to appropriately communicate these VUSs are becoming established, recognizing that an increased VUS rate is a by-product of increased diagnostic yield.[4,19,20] Nevertheless, ultimately classifying variants as pathogenic or benign is important for both clinicians and patients. The total number of variants to classify is daunting: in this study, 0.7 new uninterpreted variants were added per patient (Figure 4), and this rate changed only modestly, even after thousands of additional patients were tested in our laboratory for the same genes (data not presented). Larger and more accurate population allele frequency databases will help classify some of these variants,[93] although in a preliminary investigation, we observed that recently available data from 60,706 exomes (Exome Aggregation Consortium, Cambridge,

MA; *http://exac.broadinstitute.org*, last accessed May 5, 2015) would have allowed only a modest fraction of our VUSs to be reclassified under the same allele frequency thresholds. Functional databases will prove even more critical over time,[78,94,95] as will registries including Prospective Registry of MultiPlex Testing (*https://connect.patientcrossroads.org/?org=prompt*, last accessed November 1, 2014) and Inherited Cancer Registry (*http://inheritedcancer.net*, last accessed November 1, 2014).

In conclusion, our data show that NGS can achieve high analytic sensitivity and specificity in comparison with traditional genetic testing methods, even for some technically challenging classes of genetic variation that make up a significant fraction of the pathogenic variants in HBOC. Interpretation concordance for *BRCA1* and *BRCA2* also was high, demonstrating that broadly available resources combined with recent guidelines can produce results similar to those of an established laboratory using a large proprietary database. Thus, gene panels can be a viable replacement for traditional tests in appropriate circumstances. The additional pathogenic variants uncovered by panel testing appeared clinically relevant, albeit with the caveat of identifying many additional VUSs. Of course, ongoing studies to more fully understand the clinical utility of these panels will be important to help evolve guidelines as laboratory technologies, interpretation methods, and public databases continually improve.

Note Added in Proof

The patient management implications of the non-BRCA1/2 findings reported herein are detailed in a separate companion article.[96]

Acknowledgments

We thank Andrew Schultz and Devika Salunke for sample processing; Curtis Kautzer, Michael Kennemer, Dan Kvitek, Angela Chou, John Major, and the Invitae laboratory staff for NGS process development and data generation; John Garcia, Blanca Herrera, Yuan-Yuan Ho, Keith Nykamp, Nila Patil, and Janita Thusberg for variant interpretation; Raluca Kurtz, Kristen Shannon, and Michele Gabree for genetic counseling expertise; Scott Topper, Swaroop Aradhya, and Krishna Pant for their valuable insights and leadership; Mark Nichol and Katherine Griffin for assistance with this manuscript; and the patients and families who consented to participate in the Massachusetts General Hospital and Stanford research programs.

S.E.L., A.W.K., J.M.F., and L.W.E. designed and oversaw this study. Y.K. and M.J.A. oversaw variant interpretation and provided scientific insight. S.E.L., S.Y., A.J.D., M.A.M., G.B.N. and K.B.J. performed data management and data analysis. S.E.L., Y.K., S.Y. and F.A.M. drafted the initial manuscript, and all authors reviewed and contributed to the final manuscript.

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.jmoldx.2015.04.009*.

References

1. Rehm HL: Disease-targeted sequencing: a cornerstone in the clinic. Nat Rev Genet 2013, 14:295–300
2. Tucker T, Marra M, Friedman JM: Massively parallel sequencing: the next big thing in genetic medicine. Am J Hum Genet 2009, 85: 294–300
3. Johansen Taber KA, Dickinson BD, Wilson M: The promise and challenges of next-generation genome sequencing for clinical care. JAMA Intern Med 2014, 174:275–280
4. Kurian AW, Hare EE, Mills MA, Kingham KE, McPherson L, Whittemore AS, McGuire V, Ladabaum U, Kobayashi Y, Lincoln SE, Cargill M, Ford JM: Clinical evaluation of a multiple-gene sequencing panel for hereditary cancer risk assessment. J Clin Oncol 2014, 32:2001–2009
5. Tung N, Battelli C, Allen B, Kaldate R, Bhatnagar S, Bowles K, Timms K, Garber JE, Herold C, Ellisen L, Krejdovsky J, DeLeonardis K, Sedgwick K, Soltis K, Roa B, Wenstrup RJ, Hartman AR: Frequency of mutations in individuals with breast cancer referred for BRCA1 and BRCA2 testing using next-generation sequencing with a 25-gene panel. Cancer 2015, 121:25–33
6. LaDuca H, Stuenkel AJ, Dolinsky JS, Keiles S, Tandy S, Pesaran T, Chen E, Gau CL, Palmaer E, Shoaepour K, Shah D, Speare V, Gandomi S, Chao E: Utilization of multigene panels in hereditary cancer predisposition testing: analysis of more than 2,000 patients. Genet Med 2014, 16:830–837
7. Maxwell KN, Wubbenhorst B, D'Andrea K, Garman B, Long JM, Powers J, Rathbun K, Stopfer JE, Zhu J, Bradbury AR, Simon MS, DeMichele A, Domchek SN, Nathanson KL: Prevalence of mutations in a panel of breast cancer susceptibility genes in BRCA1/2-negative patients with early-onset breast cancer. Genet Med 2014, [Epub ahead of print], doi:10.1038/gim.2014.176
8. Couch FJ, Hart SN, Sharma P, Toland AE, Wang X, Miron P, et al: Inherited mutations in 17 breast cancer susceptibility genes among a large triple-negative breast cancer cohort unselected for family history of breast cancer. J Clin Oncol 2015, 33:304–311
9. Minion LE, Dolinsky JS, Chase DM, Dunlop CL, Chao EC, Monk BJ: Hereditary predisposition to ovarian cancer, looking beyond BRCA1/BRCA2. Gynecol Oncol 2015, 137:86–92
10. Walsh T, Casadei S, Lee MK, Pennil CC, Nord AS, Thornton AM, Roeb W, Agnew KJ, Stray SM, Wickramanayake A, Norquist B, Pennington KP, Garcia RL, King M-C, Swisher EM: Mutations in 12 genes for inherited ovarian, fallopian tube, and peritoneal carcinoma identified by massively parallel sequencing. Proc Natl Acad Sci U S A 2011, 108:18032–18037
11. Castéra L, Krieger S, Rousselin A, Legros A, Baumann J-J, Bruet O, Brault B, Fouillet R, Goardon N, Letac O, Baert-Desurmont S, Tinat J, Bera O, Dugast C, Berthet P, Polycarpe F, Layet V, Hardouin A, Frébourg T, Vaur D: Next-generation sequencing for the diagnosis of hereditary breast and ovarian cancer using genomic capture targeting multiple candidate genes. Eur J Hum Genet 2014, 22:1305–1313
12. Cragun D, Radford C, Dolinsky JS, Caldwell M, Chao E, Pal T: Panel-based testing for inherited colorectal cancer: a descriptive study of clinical testing performed by a US laboratory. Clin Genet 2014, 86: 510–520
13. Chong HK, Wang T, Lu HM, Seidler S, Lu H, Keiles S, Chao EC, Stuenkel AJ, Li X, Elliott AM: The validation and clinical implementation of BRCAplus: a comprehensive high-risk breast cancer diagnostic assay. PLoS One 2014, 9:e97408

14. Bosdet IE, Docking TR, Butterfield YS, Mungall AJ, Zeng T, Coope RJ, Yorida E, Chow K, Bala M, Young SS, Hirst M, Birol I, Moore RA, Jones SJ, Marra MA, Holt R, Karsan A: A clinically validated diagnostic second-generation sequencing assay for detection of hereditary BRCA1 and BRCA2 mutations. J Mol Diagn 2013, 15:796–809
15. Stoffel EM, Mangu PB, Gruber SB, Hamilton SR, Kalady MF, Lau MWY, Lu KH, Roach N, Limburg PJ: Hereditary colorectal cancer syndromes: American Society of Clinical Oncology Clinical Practice Guideline endorsement of the familial risk–colorectal cancer: European Society for Medical Oncology Clinical Practice Guidelines. J Clin Oncol 2015, 33:209–217
16. Gargis AS, Kalman L, Berry MW, Bick DP, Dimmock DP, Hambuch T, et al: Assuring the quality of next-generation sequencing in clinical laboratory practice. Nat Biotechnol 2012, 30:1033–1036
17. Rehm HL, Bale SJ, Bayrak-Toydemir P, Berg JS, Brown KK, Deignan JL, Friez MJ, Funke BH, Hegde MR, Lyon E; Working Group of the American College of Medical Genetics and Genomics Laboratory Quality Assurance Committee: Hartman clinical laboratory standards for next-generation sequencing. Genet Med 2013, 15:733–747
18. Hall MJ, Forman AD, Pilarski R, Wiesner G, Giri VN: Gene panel testing for inherited cancer risk. J Natl Compr Canc Netw 2014, 12:1339–1346
19. Mauer CB, Pirzadeh-Miller SM, Robinson LD, Euhus DM: The integration of next-generation sequencing panels in the clinical cancer genetics practice: an institutional experience. Genet Med 2014, 16:407–412
20. Fecteau H, Vogel KJ, Hanson K, Morrill-Cornelius S: The evolution of cancer risk assessment in the era of next generation sequencing. J Genet Couns 2014, 23:633–639
21. Robson M: Multigene panel testing: planning the next generation of research studies in clinical cancer genetics. J Clin Oncol 2014, 32:1987–1989
22. Grady D, Pollack A: Finding Risks, Not Answers, in Expanding Array of Gene Tests. New York Times 2014 Sep 23, p. A17
23. Rahman N: Mainstreaming genetic testing of cancer predisposition genes. Clin Med 2014, 14:436–439
24. Harismendy O, Ng PC, Strausberg RL, Wang X, Stockwell TB, Beeson KY, Schork NJ, Murray SS, Topol EJ, Levy S, Frazer KA: Evaluation of next generation sequencing platforms for population targeted sequencing studies. Genome Biol 2009, 10:R32
25. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A 1977, 74:5463–5467
26. Barrois M, Bièche I, Mazoyer S, Champème MH, Bressac-de Paillerets B, Lidereau R: Real-time PCR-based gene dosage assay for detecting BRCA1 rearrangements in breast–ovarian cancer families. Clin Genet 2004, 65:131–136
27. Hogervorst FB, Nederlof PM, Gille JJ, McElgunn CJ, Grippeling M, Pruntel R, Regnerus R, van Welsem T, van Spaendonk R, Menko FH, Kluijt I, Dommering C, Verhoef S, Schouten JP, van't Veer LJ, Pals G: Large genomic deletions and duplications in the BRCA1 gene identified by a novel quantitative method. Cancer Res 2003, 63:1449–1453
28. Frolov A, Prowse AH, Vanderveer L, Bove B, Wu H, Godwin AK: DNA array-based method for detection of large rearrangements in the BRCA1 gene. Cancer 2002, 35:232–241
29. Plon SE, Eccles DM, Easton D, Foulkes WD, Genuardi M, Greenblatt MS, Hogervorst FB, Hoogerbrugge N, Spurdle AB, Tavtigian SV; IARC Unclassified Genetic Variants Working Group: Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results. Hum Mutat 2008, 29:1282–1291
30. Cook-Deegan R, Conley JM, Evans JP, Vorhaus D: The next controversy in genetic testing: clinical data as trade secrets? Eur J Hum Genet 2013, 21:585–588
31. Cook-Deegan R, Niehaus A: After myriad: genetic testing in the wake of recent Supreme Court decisions about gene patents. Curr Genet Med Rep 2014, 2:223–241
32. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL: Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 2015, 17:405–423
33. Daly MB, Pilarski R, Axilbund JE, Buys SS, Crawford B, Friedman S, Garber JE, Horton C, Kaklamani V, Klein C, Kohlmann W, Kurian A, Litton J, Madlensky L, Marcom PK, Merajver SD, Offit K, Pal T, Pasche B, Reiser G, Shannon KM, Swisher E, Voian NC, Weitzel JN, Whelan A, Wiesner GL, Dwyer MA, Kumar R: Genetic/familial high-risk assessment: breast and ovarian, version 1.2014. J Natl Compr Canc Netw 2014, 12:1326–1338
34. Zook JM, Chapman B, Wang J, Mittelman D, Hofmann O, Hide W, Salit M: Integrating human sequence data sets provides a resource of benchmark SNP and indel genotype calls. Nat Biotechnol 2014, 32:246–251
35. Abecasis GR, Cherny SS, Cookson WO, Cardon LR: Merlin: rapid analysis of dense genetic maps using sparse gene flow trees. Nat Genet 2002, 30:97–101
36. Roach JC, Glusman G, Smit AF, Huff CD, Hubley R, Shannon PT, Rowen L, Pant KP, Goodman N, Bamshad M, Shendure J, Drmanac R, Jorde LB, Hood L, Galas DJ: Analysis of genetic inheritance in a family quartet by whole-genome sequencing. Science 2010, 328:636–639
37. Chen S, Parmigiani G: Meta-analysis of BRCA1 and BRCA2 penetrance. J Clin Oncol 2007, 25:1329–1333
38. Mavaddat N, Peock S, Frost D, Ellis S, Platte R, Fineberg E, Evans DG, Izatt L, Eeles RA, Adlard J, Davidson R, Eccles D, Cole T, Cook J, Brewer C, Tischkowitz M, Douglas F, Hodgson S, Walker L, Porteous ME, Morrison PJ, Side LE, Kennedy MJ, Houghton C, Donaldson A, Rogers MT, Dorkins H, Miedzybrodzka Z, Gregory H, Eason J, Barwell J, McCann E, Murray A, Antoniou AC, Easton DF; EMBRACE: Cancer risks for BRCA1 and BRCA2 mutation carriers: results from prospective analysis of EMBRACE. J Natl Cancer Inst 2013, 105:812–822
39. Pilarski R, Eng C: Will the real Cowden syndrome please stand up (again)? expanding mutational and clinical spectra of the PTEN hamartoma tumour syndrome. J Med Genet 2004, 41:323–326
40. Tan MH, Mester JL, Ngeow J, Rybicki LA, Orloff MS, Eng C: Lifetime cancer risks in individuals with germline PTEN mutations. Clin Cancer Res 2012, 18:400–407
41. Gonzalez KD, Noltner KA, Buzin CH, Gu D, Wen-Fong CY, Nguyen VQ, Han JH, Lowstuter K, Longmate J, Sommer SS, Weitzel JN: Beyond Li Fraumeni syndrome: clinical characteristics of families with p53 germline mutations. J Clin Oncol 2009, 27:1250–1256
42. Hearle N, Schumacher V, Menko FH, Olschwang S, Boardman LA, Gille JJ, Keller JJ, Westerman AM, Scott RJ, Lim W, Trimbath JD, Giardiello FM, Gruber SB, Offerhaus GJ, de Rooij FW, Wilson JH, Hansmann A, Möslein G, Royer-Pokora B, Vogel T, Phillips RK, Spigelman AD, Houlston RS: Frequency and spectrum of cancers in the Peutz-Jeghers syndrome. Clin Cancer Res 2006, 12:3209–3215
43. Lim W, Olschwang S, Keller JJ, Westerman AM, Menko FH, Boardman LA, Scott RJ, Trimbath J, Giardiello FM, Gruber SB, Gille JJ, Offerhaus GJ, de Rooij FW, Wilson JH, Spigelman AD, Phillips RK, Houlston RS: Relative frequency and morphology of cancers in STK11 mutation carriers. Gastroenterology 2004, 126:1788–1794
44. Kaurah P, MacMillan A, Boyd N, Senz J, De Luca A, Chun N, et al: Founder and recurrent CDH1 mutations in families with hereditary diffuse gastric cancer. JAMA 2007, 297:2360–2372
45. Pharoah PD, Guilford P, Caldas C; International Gastric Cancer Linkage Consortium: Incidence of gastric cancer and breast cancer in CDH1 (E-cadherin) mutation carriers from hereditary diffuse gastric cancer families. Gastroenterology 2001, 121:1348–1353
46. Rahman N, Seal S, Thompson D, Kelly P, Renwick A, Elliott A, Reid S, Spanova K, Barfoot R, Chagtai T, Jayatilake H, McGuffog L,

Hanks S, Evans DG, Eccles D; Breast Cancer Susceptibility Collaboration (UK), Easton DF, Stratton MR: PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. Nat Genet 2007, 39:165−167
47. Antoniou AC, Casadei S, Heikkinen T, Barrowdale D, Pylkäs K, Roberts J, et al: Breast-cancer risk in families with mutations in PALB2. N Engl J Med 2014, 371:497−506
48. Weischer M, Bojesen SE, Tybjaerg-Hansen A, Axelsson CK, Nordestgaard BG: Increased risk of breast cancer associated with CHEK2*1100delC. J Clin Oncol 2007, 25:57−63
49. Seal S, Thompson D, Renwick A, Elliott A, Kelly P, Barfoot R, Chagtai T, Jayatilake H, Ahmed M, Spanova K, North B, McGuffog L, Evans DG, Eccles D; Breast Cancer Susceptibility Collaboration (UK), Easton DF, Stratton MR, Rahman N: Truncating mutations in the Fanconi anemia J gene BRIP1 are low-penetrance breast cancer susceptibility alleles. Nat Genet 2006, 38:1239−1241
50. Meindl A, Hellebrand H, Wiek C, Erven V, Wappenschmidt B, Niederacher D, Freund M, Lichtner P, Hartmann L, Schaal H, Ramser J, Honisch E, Kubisch C, Wichmann HE, Kast K, Deissler H, Engel C, Müller-Myhsok B, Neveling K, Kiechle M, Mathew CG, Schindler D, Schmutzler RK, Hanenberg H: Germline mutations in breast and ovarian cancer pedigrees establish RAD51C as a human cancer susceptibility gene. Nat Genet 2010, 42:410−414
51. Bogdanova N, Feshchenko S, Schürmann P, Waltes R, Wieland B, Hillemanns P, Rogov YI, Dammann O, Bremer M, Karstens JH, Sohn C, Varon R, Dörk T: Nijmegen breakage syndrome mutations and risk of breast cancer. Int J Cancer 2008, 122:802−806
52. Seemanová E, Jarolim P, Seeman P, Varon R, Digweed M, Swift M, Sperling K: Cancer risk of heterozygotes with the NBN founder mutation. J Natl Cancer Inst 2007, 99:1875−1880
53. Ahmed M, Rahman N: ATM and breast cancer susceptibility. Oncogene 2006, 25:5906−5911
54. Win AK, Lindor NM, Young JP, Macrae FA, Young GP, Williamson E, Parry S, Goldblatt J, Lipton L, Winship I, Leggett B, Tucker KM, Giles GG, Buchanan DD, Clendenning M, Rosty C, Arnold J, Levine AJ, Haile RW, Gallinger S, Le Marchand L, Newcomb PA, Hopper JL, Jenkins MA: Risks of primary extracolonic cancers following colorectal cancer in lynch syndrome. J Natl Cancer Inst 2012, 104:1363−1372
55. Jiang L, Zhang C, Li Y, Yu X, Zheng J, Zou P, Li Y, Bin X, Lu J, Zhou Y: A non-synonymous polymorphism Thr115Met in the EpCAM gene is associated with an increased risk of breast cancer in Chinese population. Breast Cancer Res Treat 2011, 126:487−495
56. Redston M, Nathanson KL, Yuan ZQ, Neuhausen SL, Satagopan J, Wong N, Yang D, Nafa D, Abrahamson J, Ozcelik H, Antin-Ozerkis D, Andrulis I, Daly M, Pinsky L, Schrag D, Gallinger S, Kaback M, King MC, Woodage T, Brody LC, Godwin A, Warner E, Weber B, Foulkes W, Offit K: The APCI1307K allele and breast cancer risk. Nat Genet 1998, 20:13−14
57. Saetrom P, Biesinger J, Li SM, Smith D, Thomas LF, Majzoub K, Rivas GE, Alluin J, Rossi JJ, Krontiris TG, Weitzel J, Daly MB, Benson AB, Kirkwood JM, O'Dwyer PJ, Sutphen R, Stewart JA, Johnson D, Larson GP: A risk variant in an miR-125b binding site in BMPR1B is associated with breast cancer pathogenesis. Cancer Res 2009, 69:7459−7465
58. Tram E, Ibrahim-Zada I, Briollais L, Knight JA, Andrulis IL, Ozcelik H: Identification of germline alterations of the mad homology 2 domain of SMAD3 and SMAD4 from the Ontario site of the breast cancer family registry (CFR). Breast Cancer Res 2011, 13:R77
59. Dean JL, McClendon AK, Hickey TE, Butler LM, Tilley WD, Witkiewicz AK, Knudsen ES: Therapeutic response to CDK4/6 inhibition in breast cancer defined by ex vivo analyses of human tumors. Cell Cycle 2012, 11:2756−2761
60. Debniak T, Górski B, Huzarski T, Byrski T, Cybulski C, Mackiewicz A, Gozdecka-Grodecka S, Gronwald J, Kowalska E, Haus O, Grzybowska E, Stawicka M, Swiec M, Urbański K, Niepsuj S, Waśko B, Góźdź S, Wandzel P, Szczylik C, Surdyka D, Rozmiarek A, Zambrano O, Posmyk M, Narod SA, Lubinski J: A common variant of CDKN2A (p16) predisposes to breast cancer. J Med Genet 2005, 42:763−765
61. Pogue-Geile KL, Chen R, Bronner MP, Crnogorac-Jurcevic T, Moyes KW, Dowen S, Otey CA, Crispin DA, George RD, Whitcomb DC, Brentnall TA: Palladin mutation causes familial pancreatic cancer and suggests a new cancer mechanism. PLoS Med 2006, 3:e516
62. Zogopoulos G, Rothenmund H, Eppel A, Ash C, Akbari MR, Hedley D, Narod SA, Gallinger S: The P239S palladin variant does not account for a significant fraction of hereditary or early onset pancreas cancer. Hum Genet 2007, 121:635−637
63. Neklason DW, Done MW, Sargent NR, Schwartz AG, Anton-Culver H, Griffin CA, Ahnen DJ, Schildkraut JM, Tomlinson GE, Strong LC, Miller AR, Stopfer JE, Burt RW: Activating mutation in MET oncogene in familial colorectal cancer. BMC Cancer 2011, 11:424
64. Lemmens I, Van de Ven WJ, Kas K, Zhang CX, Giraud S, Wautot V, Buisson N, De Witte K, Salandre J, Lenoir G, Pugeat M, Calender A, Parente F, Quincey D, Gaudray P, De Wit MJ, Lips CJ, Höppener JW, Khodaei S, Grant AL, Weber G, Kytölä S, Teh BT, Farnebo F, Thakker RV; European Consortium on MEN1: Identification of the multiple endocrine neoplasia type 1 (MEN1) gene. Hum Mol Genet 1997, 6:1177−1183
65. Machens A, Lorenz K, Sekulla C, Höppner W, Frank-Raue K, Raue F, Dralle H: Molecular epidemiology of multiple endocrine neoplasia 2: implications for RET screening in the new millenium. Eur J Endocrinol 2013, 168:307−314
66. Lee SJ, Do IG, Lee J, Kim KM, Jang J, Sohn I, Kang WK: Gastric cancer (GC) patients with hedgehog pathway activation: PTCH1 and GLI2 as independent prognostic factors. Target Oncol 2013, 8:271−280
67. Kong W, He L, Richards EJ, Challa S, Xu CX, Permuth-Wey J, Lancaster JM, Coppola D, Sellers TA, Djeu JY, Cheng JQ: Upregulation of miRNA-155 promotes tumour angiogenesis by targeting VHL and is associated with poor prognosis and triple-negative breast cancer. Oncogene 2014, 33:679−689
68. Rennert G, Lejbkowicz F, Cohen I, Pinchev M, Rennert HS, Barnett-Griness O: MUTYH mutation carriers have increased breast cancer risk. Cancer 2012, 118:1989−1993
69. Win AK, Dowty JG, Cleary SP, Kim H, Buchanan DD, Young JP, Clendenning M, Rosty C, MacInnis RJ, Giles GG, Boussioutas A, Macrae FA, Parry S, Goldblatt J, Baron JA, Burnett T, Le Marchand L, Newcomb PA, Haile RW, Hopper JL, Cotterchio M, Gallinger S, Lindor NM, Tucker KM, Winship IM, Jenkins MA: Risk of colorectal cancer for carriers of mutations in MUTYH, with and without a family history of cancer. Gastroenterology 2014, 146:1208−1211
70. Lubbe SJ, Di Bernardo MC, Chandler IP, Houlston RS: Clinical implications of the colorectal cancer risk associated with MUTYH mutation. J Clin Oncol 2009, 27:3975−3980
71. Bentley DR, Balasubramanian S, Swerdlow HP, Smith GP, Milton J, Brown CG, et al: Accurate whole human genome sequencing using reversible terminator chemistry. Nature 2008, 456:53−59
72. Church DM, Schneider VA, Graves T, Auger K, Cunningham F, Bouk N, et al: Modernizing reference genome assemblies. PLoS Biol 2011, 9:e1001091
73. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA: The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010, 20:1297−1303
74. Garrison E, Marth G: Haplotype-Based Variant Detection from Short-Read Sequencing. arXiv 2012:1207.3907v2 [q-bio.GN] http://arxiv.org/abs/1207.3907
75. Jacobs KB, Paul JS, Nilsen GB, Hart RK, Johnson M, Sorenson J, Cargill M, Lincoln SE: CNVitae−accurate detection of small and large copy number events from DNA capture sequence data.

Presented at the Cold Spring Harbor Biology of Genomes Meeting, 2013 May 7-11, Cold Spring Harbor, NY. Abstract 157.
76. Alkan C, Coe BP, Eichler EE: Genome structural variation discovery and genotyping. Nat Rev Genet 2011, 12:363−376
77. Nord AS, Lee M, King M-C, Walsh T: Accurate and exact CNV identification from targeted high-throughput sequence data. BMC Genomics 2011, 12:184
78. Powers MP, Anderson M, Garcia J, Nykamp K, Monzon F, Topper S: Development of a novel score based system for germline variant interpretation of clinical next-generation sequencing data. Presented at the Association for Molecular Pathology Annual Meeting, 2014 November 12-15, National Harbor, MD. J Mol Diagn 2014, 16:699, Abstract G30
79. Béroud C, Hamroun D, Collod-Béroud G, Boileau C, Soussi T, Claustres M: UMD (Universal Mutation Database): 2005 update. Hum Mutat 2005, 26:184−191
80. Kohonen-Corish MR, Macrae F, Genuardi M, Aretz S, Bapat B, Bernstein IT, Burn J, Cotton RG, den Dunnen JT, Frebourg T, Greenblatt MS, Hofstra R, Holinski-Feder E, Lappalainen I, Lindblom A, Maglott D, Møller P, Morreau H, Möslein G, Sijmons R, Spurdle AB, Tavtigian S, Tops CM, Weber TK, de Wind N, Woods MO; Contributors to the InSiGHT-HVP Workshop: Deciphering the colon cancer genes: report of the InSiGHT-Human Variome Project Workshop, UNESCO, Paris 2010. Hum Mutat 2011, 32:491−494
81. 1000 Genomes Project Consortium, Abecasis GR, Auton A, Brooks LD, DePristo MA, Durbin RM, Handsaker RE, Kang HM, Marth GT, McVean GA: An integrated map of genetic variation from 1,092 human genomes. Nature 2012, 491:56−65
82. Eggington JM, Bowles KR, Moyes K, Manley S, Esterling L, Sizemore S, Rosenthal E, Theisen A, Saam J, Arnell C, Pruss D, Bennett J, Burbidge LA, Roa B, Wenstrup RJ: A comprehensive laboratory-based program for classification of variants of uncertain significance in hereditary cancer genes. Clin Genet 2014, 86:229−237
83. McAdam AJ: Discrepant analysis: how can we test a test? J Clin Microbiol 2000, 38:2027−2029
84. Landrum MJ, Lee JM, Riley GR, Jang W, Rubinstein WS, Church DM, Maglott DR: ClinVar: public archive of relationships among sequence variation and human phenotype. Nucleic Acids Res 2014, 42(Database issue):387−406
85. Hall MJ, Reid JE, Wenstrup RJ: Prevalence of BRCA1 and BRCA2 mutations in women with breast carcinoma in situ and referred for genetic testing. Cancer Prev Res (Phila) 2010, 3:1579−1585
86. Aradhya S, Lewis R, Bonaga T, Nwokekeh N, Stafford A, Boggs B, Hruska K, Smaoui N, Compton JG, Richard G, Suchy S: Exon-level array CGH in a large clinical cohort demonstrates increased sensitivity of diagnostic testing for mendelian disorders. Genet Med 2012, 14:594−603
87. Retterer K, Scuffins J, Schmidt D, Lewis R, Pineda-Alvarez D, Stafford A, Schmidt L, Warren S, Gibellini F, Kondakova A, Blair A, Bale S, Matyakhina L, Meck J, Aradhya S, Haverfield E: Assessing copy number from exome sequencing and exome array CGH based on CNV spectrum in a large clinical cohort. Genet Med 2015, 17:623−629
88. O'Rawe J, Jiang T, Sun G, Wu Y, Wang W, Hu J, Bodily P, Tian L, Hakonarson H, Johnson WE, Wei Z, Wang K, Lyon GJ: Low concordance of multiple variant-calling pipelines: practical implications for exome and genome sequencing. Genome Med 2013, 5:387−406
89. Jia H, Guo Y, Zhao W, Wang K: Long-range PCR in next-generation sequencing: comparison of six enzymes and evaluation on the MiSeq sequencer. Sci Rep 2014, 4:387−406
90. MacArthur DG, Balasubramanian S, Frankish A, Huang N, Morris J, Walter K, et al: A systematic survey of loss-of-function variants in human protein-coding genes. Science 2012, 335:823−828
91. Strom SP, Lee H, Das K, Vilain E, Nelson SF, Grody WW, Deignan JL: Assessing the necessity of confirmatory testing for exome-sequencing results in a clinical molecular diagnostic laboratory. Genet Med 2014, 16:510−515
92. Daly MB, Pilarski R, Axilbund JE, Berry M, Buys SS, Crawford B, et al: National Comprehensive Cancer Network clinical practice guidelines in oncology. Genetic/familial high-risk assessment: breast and ovarian. Version 1.2015. [Internet]. Available at http://www.nccn.org/professionals/physician_gls/pdf/genetics_screening.pdf
93. Shearer AE, Eppsteiner RW, Booth KT, Ephraim SS, Gurrola J 2nd, Simpson A, Black-Ziegelbein EA, Joshi S, Ravi H, Giuffre AC, Happe S, Hildebrand MS, Azaiez H, Bayazit YA, Erdal ME, Lopez-Escamez JA, Gazquez I, Tamayo ML, Gelvez NY, Leal GL, Jalas C, Ekstein J, Yang T, Usami S, Kahrizi K, Bazazzadegan N, Najmabadi H, Scheetz TE, Braun TA, Casavant TL, LeProust EM, Smith RJ: Utilizing ethnic-specific differences in minor allele frequency to recategorize reported pathogenic deafness variants. Am J Hum Genet 2014, 94:387−406
94. Spurdle AB, Healey S, Devereau A, Hogervorst FB, Monteiro AN, Nathanson KL, Radice P, Stoppa-Lyonnet D, Tavtigian S, Wappenschmidt B, Couch FJ, Goldgar DE; ENIGMA: ENIGMA—Evidence-Based Network for the Interpretation of Germline Mutant Alleles: an international initiative to evaluate risk and clinical significance associated with sequence variation in BRCA1 and BRCA2 genes. Hum Mutat 2012, 33:387−406
95. Couch FJ, Rasmussen LJ, Hofstra R, Monteiro AN, Greenblatt MS, de Wind N; IARC Unclassified Genetic Variants Working Group: Assessment of functional effects of unclassified genetic variants. Hum Mutat 2008, 29:1314−1326
96. Desmond A, Kurian AW, Gabree M, Mills MA, Anderson MJ, Kobayashi Y, Horick N, Yang S, Shannon KM, Tung N, Ford JM, Lincoln SE, Ellisen LW: Clinical actionability of multi-gene panel testing for hereditary breast and ovarian cancer risk assessment. JAMA Oncol 2015, [Epub ahead of print], doi:10.1001/jamaoncol.2015.2690