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ORIGINAL REPORT

Clinical Evaluation of a Multiple-Gene Sequencing Panel for Hereditary Cancer Risk Assessment

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Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

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ABSTRACT

Purpose
Multiple-gene sequencing is entering practice, but its clinical value is unknown. We evaluated the performance of a customized germline-DNA sequencing panel for cancer-risk assessment in a representative clinical sample.

Methods
Patients referred for clinical *BRCA1/2* testing from 2002 to 2012 were invited to donate a research blood sample. Samples were frozen at −80° C, and DNA was extracted from them after 1 to 10 years. The entire coding region, exon-intron boundaries, and all known pathogenic variants in other regions were sequenced for 42 genes that had cancer risk associations. Potentially actionable results were disclosed to participants.

Results
In total, 198 women participated in the study: 174 had breast cancer and 57 carried germline *BRCA1/2* mutations. *BRCA1/2* analysis was fully concordant with prior testing. Sixteen pathogenic variants were identified in *ATM*, *BLM*, *CDH1*, *CDKN2A*, *MUTYH*, *MLH1*, *NBN*, *PRSS1*, and *SLX4* among 141 women without *BRCA1/2* mutations. Fourteen participants carried 15 pathogenic variants, warranting a possible change in care; they were invited for targeted screening recommendations, enabling early detection and removal of a tubular adenoma by colonoscopy. Participants carried an average of 2.1 variants of uncertain significance among 42 genes.

Conclusion
Among women testing negative for *BRCA1/2* mutations, multiple-gene sequencing identified 16 potentially pathogenic mutations in other genes (11.4%; 95% CI, 7.0% to 17.7%), of which 15 (10.6%; 95% CI, 6.5% to 16.9%) prompted consideration of a change in care, enabling early detection of a precancerous colon polyp. Additional studies are required to quantify the penetrance of identified mutations and determine clinical utility. However, these results suggest that multiple-gene sequencing may benefit appropriately selected patients.

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INTRODUCTION

Clinical genetic testing for cancer-risk assessment has become widespread over the last two decades, with evidence-based testing guidelines for hereditary breast and ovarian cancer (*BRCA1* and *BRCA2*; *BRCA1/2*), Lynch syndrome (*MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM*), familial adenomatous polyposis (*APC*), hereditary diffuse gastric cancer (*CDH1*), Li-Fraumeni syndrome (*TP53*), Cowden's syndrome (*PTEN*), and a few other conditions.[1-4] Cancer genetic counseling and risk-reducing interventions have accordingly been developed for high penetrance, autosomal dominant conditions. Most of these interventions, especially prophylactic surgery, are excessive for carriers of mutations that have uncertain pathogenicity.[5-7] Recently, next-generation technology has enabled massively parallel sequencing at low cost, and panels of multiple cancer-associated genes are newly available for clinical use.[8,9]

Despite these advances in technology, a critical knowledge deficit remains about the clinical value of multiple-gene panels for cancer susceptibility. Major questions include how many and which genes to sequence, whether results are sufficiently understood to guide intervention, and how best to counsel patients about variants of low or moderate penetrance.[8,10-12] We designed a customized germline sequencing panel of 42 cancer-associated genes and evaluated its information yield among women referred for clinical evaluation of hereditary breast

and ovarian cancer. Specifically, we aimed to assess the concordance of results with prior clinical sequencing, the prevalence of potentially actionable results, and the downstream effects on cancer screening and risk-reduction.

METHODS

Participant Accrual

Patients were eligible to participate if they were female, at least 18 years old, and had undergone clinical *BRCA1/2* testing at the Stanford University Clinical Cancer Genetics program from 2002 to 2012. The criteria for clinical genetic counseling and *BRCA1/2* testing were those accepted by insurance carriers, based on guidelines of the National Comprehensive Cancer Network at the time of evaluation. Some patients had a personal history of breast and/or ovarian cancer; some had a family history of breast, ovarian, pancreatic, and/or prostate cancers; and some had both personal and family history of such cancers.[2,3] Since 2002, all patients undergoing clinical *BRCA1/2* testing (for an identified familial mutation; the Ashkenazi Jewish three-founder mutations panel; full sequencing with analysis of five common *BRCA1* rearrangements; and/or full sequencing with comprehensive rearrangement analysis, depending on the indication and year) were offered participation in a study approved by the Stanford University institutional review board (IRB). Patients were informed that participation would consist of donating two 5-mL tubes of blood for clinical *BRCA1/2* testing and that they might be contacted about participation in future research. Blood specimens were frozen at −80° C and were linked to demographic, clinical, and genetic data stored in a secure research database.[13-15]

Gene Selection

Genes were selected through a review of published literature. Most genes had a reported breast cancer association but some were associated with other cancer syndromes or DNA repair pathways, rendering a breast cancer–risk association plausible (Table 1).

Sequencing

Sequencing was performed at InVitae (San Francisco, CA), a clinical laboratory improvement amendments (CLIA) –approved laboratory. Two micrograms of genomic DNA per patient were sheared on a Covaris E220 sonicator (Woburn, MA) to 250 base pair (bp) mean fragment size. Genomic DNA was quantified and assessed for quality using the Life Technologies Quant-iT PicoGreen double-strand DNA assay kit (Carlsbad, CA). The entire coding region, exon-intron boundaries (± 10 bp), and other regions containing known pathogenic variants were targeted and captured using Agilent SureSelect custom RNA probes (Santa Clara, CA) and Integrated DNA Technologies xGen Lockdown custom DNA probes (Coral, IL). Sequencing libraries were constructed using the Agilent SureSelectXT protocol and were quantified with the KAPA Biosystems Library Quantification Kit (Woburn, MA). These steps were performed in an automated fashion using the Agilent Bravo automated liquid-handling platform. Quantified libraries were sequenced on the Illumina MiSeq platform (San Diego, CA) using the 2 × 151 bp configuration to at least 400× average coverage. Bioinformatics and data quality control followed the Genome Analysis Toolkit best-practices (Broad Institute, Cambridge, MA), with additional algorithms to detect larger insertions, deletions, and duplications.[53] *PMS2* exons 12 to 15 were excluded from analysis because of high homology to the known pseudogene. Deletion/duplication data were not available for 54 samples sequenced before the development of calibration standards used by the algorithm.

Variant Classification

Sequence variants and large insertions and deletions were classified according to the American College of Medical Genetics (ACMG) guidelines for variant interpretation.[54] Variants were classified as pathogenic or likely pathogenic (collectively termed, pathogenic) if they conferred a truncating, initiation codon or splice donor/acceptor effect, if functional data demonstrated an effect on protein function relevant to disease phenotype, or if pathogenicity was otherwise demonstrated in published literature. If no functional data were

Table 1. Sequenced Genes and Criteria for Their Inclusion

Fully Sequenced Genes (n = 42)	Breast Cancer Relative Risk (or other inclusion criterion)	References
APC	Unknown (causes Familial Adenomatous Polyposis)	Redston et al[16]
ATM	1.5-3.8	Renwick et al[17]
BLM	1.2-3.3	Broberg et al[18]
BMPR1A	1.3	Saetrom et al[19]
BRCA1	4.0-7.0	Chen et al[20]
BRCA2	4.0-7.0	Chen et al[21]
BRIP1	1.2-3.2	Seal et al[22]
CDH1	5.9-7.3	Kaurah et al,[23] Pharoah et al[24]
CDK4	Unknown (functions as DNA repair gene)	Dean et al[25]
CDKN2A	1.1-1.7	Debniak et al[26]
EPCAM	1.2-1.6	Jiang et al[27]
FANCA	0.9-1.0	Barroso et al[28]
FANCB	0.8-1.2	Barroso et al[28]
FANCC	1.0-1.4	Barroso et al[28]
FANCD2	1.1-1.7	Barroso et al[28]
FANCE	0.9-1.2	Barroso et al[28]
FANCF	0.9-1.4	Barroso et al[28]
FANCG	0.8-1.0	Barroso et al[28]
FANCI	0.8-1.1	Barroso et al[28]
FANCL	1.0-1.1	Barroso et al[28]
LIG4	0.4-1.0	Kuschel et al[29]
MEN1	Unknown (causes multiple endocrine neoplasia 1)	Lemmens et al[30]
MET	Unknown (associated with papillary renal cell carcinoma risk)	Neklason et al[31]
MLH1	0.2-2.0	Win et al[32]
MSH2	1.2-3.7	Win et al[32]
MSH6	0-13	Win et al[32]
MUTYH	1.0-3.4	Rennert et al[33]
NBN	1.4-6.6	Bogdanova et al,[34] Seemanová et al,[35] Zhang et al[36]
PALB2	1.4-3.9	Rahman et al[37]
PALLD	Unknown (associated with pancreatic cancer risk)	Pogue-Geile et al[38]
PMS2	Unknown (causes Lynch syndrome)	Win[32]
PRSS1	0.7-1.6	Wagner et al[39]
PTCH1	Unknown (associated with basal cell nevus syndrome and glioblastoma)	Lee et al[40]
PTEN	2.0-5.0	Pilarski et al,[41] Tan et al[42]
RAD51C	1.5-7.8	Meindl et al[43]
RET	Unknown (causes multiple endocrine neoplasia 2)	Machens et al[44]
SLX4	1.0-2.0	Landwehr et al,[45] Shah et al[46]
SMAD4	Unknown (mutated in breast tumors)	Tram et al[47]
SPINK1	Unknown (mutated in breast tumors)	Soon et al[48]
STK11	2.0-4.0	Hearle et al,[49] Lim et al[50]
TP53	4.3-9.3	Gonzalez et al[51]
VHL	Unknown (mutated in breast tumors)	Kong et al[52]

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available, missense, silent, and intronic variants were classified as variants of uncertain significance (VUS), benign or likely benign based on allele frequency in the 1,000 Genomes Study,[55] dbSNP,[56] or the Exome Variant Server.[57] Also, published literature on *BRCA1/2* VUS was reviewed to classify those variants further as benign or likely benign following the ACMG guidelines. For pathogenic variants, we then reviewed published literature and practice guidelines to assign a clinical status of potentially actionable versus not actionable. We defined potentially actionable results as pathogenic variants that either cause recognized hereditary cancer syndromes, such as Lynch syndrome or hereditary diffuse gastric cancer,[58] or have a published association with a two-fold or greater relative risk of breast cancer compared with that of an average woman, under which circumstance guidelines recommend annual screening with breast magnetic resonance imaging (MRI) and mammograms.[59-61] Participants with potentially actionable results were contacted by telephone, were invited to a genetic counseling visit, and were offered a CLIA-approved test to confirm research findings. Cancer screening and prevention recommendations were consistent with clinical practice guidelines focused on the estimated magnitude of cancer risk, given the absence of gene-specific guidelines for many of the sequenced genes.[2,59,60,62,63] Participants were not notified about results that do not currently affect care recommendations, including absence of sequence abnormalities, pathogenic variants considered not actionable, or variants of uncertain significance.[2,58,59,60,63]

Statistical Analysis

Participant characteristics and sequencing results were tabulated, with descriptive statistics including medians, means, and standard deviations for continuous data and proportions with 95% CI for categoric data. Proportions were compared using the χ^2 statistic. All *P* values are two-tailed.

RESULTS

Participant Characteristics

From January 1, 2001 to June 30, 2013, 1,805 patients underwent clinical *BRCA1/2* testing in the Stanford Clinical Cancer Genetics program. Six hundred fifty-four patients (36.2%) donated a research blood sample during clinical testing. From 654 research samples, 198 were randomly selected for study participation; 174 participants had breast cancer, and 57 carried a *BRCA1/2* mutation. Participants were diverse in age and race/ethnicity and were generally representative of the clinical population from which they were recruited (Table 2).

Pathogenic Variants in BRCA1 and BRCA2

Before study enrollment, 57 participants were known to carry 59 pathogenic variants in *BRCA1/2* as determined by standard clinical testing (Myriad Genetics, Salt Lake City, UT). The remaining 141 participants had tested negative for *BRCA1/2* mutations. Fifty-seven of these 59 mutations in *BRCA1/2* were confirmed by the gene panel. Of the two others, one was detected but interpreted as a VUS because the information in the literature did not meet ACMG criteria for pathogenicity.[54] The other was a large insertion in one of the 54 samples for which del/dup analysis was not performed in the panel test. Considering the assays performed, *BRCA1/2* analysis was

Table 2. Characteristics of the Study Participants Compared With the Larger Clinical Source Population

Characteristic	Study Participants (n = 198)		*BRCA1/2* Test at Stanford, 2001-2013 (n = 1,805)	
	No. of Participants	%	No. of Participants	%
Age, years				
Median	48		46	
Race/ethnicity				
NH white, Ashkenazi Jew	7	3.5	102	5.6
NH white, not Ashkenazi Jew	140	70.7	1,205	66.7
NH black	3	1.5	30	1.7
NH Asian/Pacific Islander	39	19.7	292	16.2
Hispanic	7	3.5	92	5.1
Unknown/other race or ethnicity*	2	1.0	84	4.6
Personal history of breast cancer				
Unilateral	138	69.7	1,139	63.1
Bilateral†	36	18.1	139	7.7
Age at first breast cancer diagnosis, years				
Median	44		44	
Personal history of ovarian cancer†	1	0.5	136	7.5
Age at ovarian cancer diagnosis, median	72		52	
Personal history of other cancer‡	18	9.1	175	9.7
Family history of breast cancer	148	74.7	1,285	71.2
Family history of ovarian cancer	52	26.3	444	24.6
BRCA1 mutation§	35	17.7	213	11.8
BRCA2 mutation	24	12.1	194	10.7
Year of first clinic visit				
Median	2008		2009	
Range	2005-2011		2001-2013	

Abbreviation: NH, non-Hispanic.
*For comparison between study participants and source population, $P = .01$ (χ^2 two-tailed test).
†For comparison between study participants and source population, $P < .001$ (χ^2 two-tailed test).
‡Other cancers were nonmelanoma skin cancer (n = 6), endometrial cancer (n = 3), melanoma (n = 2), colon cancer (n = 1), leukemia (n = 1), lymphoma (n = 1), pancreatic cancer (n = 1), salivary gland cancer (n = 1), thyroid cancer (n = 1), and unknown primary cancer (n = 1).
§For comparison between study participants and source population, $P = .02$ (χ^2 two-tailed test).

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Table 3. Pathogenic Variants in Genes Other Than *BRCA1* or *BRCA2*, With Participant Characteristics and Clinical Decisions (potentially actionable or not)

Study ID	Race/ Ethnicity	Cancer Site/Subtype	Age at Diagnosis (years)	*BRCA1/2* Mutation	Affected Gene	Variant Name	Variant Effect	Protein Change	Family Cancer History*	Clinical Decision	Reference
LS221	NH white	Breast, bilateral: ER/PR+, HER2−	44, 55	None	*ATM*	NM_000051.3:c.1402_1403delAA	Frameshift	Lys468GlufsX18	Breast (45); prostate (n = 3; 60s)	PA	Buzin et al[64]
LS294	NH white	Breast: ER−/PR−/HER2−; endometrial	35, 46	None	*MLH1*	NM_000249.3:c.2190delT	Frameshift	Pro731LeufsX52	Breast (70); neuroblastoma (10); ovary (55); pancreas (55)	PA	Rouleau et al[65]
LS305	Filipino	Breast: ER/PR+, HER2−	43	None	*PRSS1*	NM_002769.4:c.346C>T	Missense	Arg116Cys	Breast (n = 3; 30s-70s); lymphoma (74); prostate (65)	PA	Szmola and Sahin-Tóth[66]
LS306	NH white	Breast, bilateral: ER/PR+, HER2−	57, 63	None	*BLM*	NM_000057.2:c.3558 + 1G>T	Splice donor	Not applicable	Breast, ovary (40s); colon (n = 3; 40s-70s); prostate (70)	PA	Novel
LS324	Filipino	Breast, bilateral: ER/PR+, HER2−	47, 55	*BRCA1*	*MUTYH*	NM_001048171.1:c.892-2A>G	Splice acceptor	Not applicable	Breast (57); ovarian (47); prostate (70)	PA	Novel
LS347	NH white	No personal cancer history	None	None	*SLX4*	NM_032444.2:c.5233_5234delGC	Frameshift	Ala1745SerfsX32	Bladder (75); breast (n = 3; 40s); colon (85)	PA	Novel
LS358	NH white	No personal cancer history	None	None	*MUTYH*	NM_001048171.1:c.1145G>A	Missense	Gly382Asp	Breast (45); colon (n = 2; 45, 65); ovarian (60)	PA	Ali et al,[67] Eliason et al[68]
LS373	Hispanic	Breast: ER/PR+, HER2−	36	None	*CDKN2A*	NM_000077.4:c.146T>C	Missense	Ile49Thr	Lung (55); unknown primary	NA	Lal et al[69]
LS380	Hispanic	Breast, bilateral: ER/PR+, ER/PR−	27, 33	None	*MUTYH*	NM_001048171.1:c.694G>T	Missense	Val232Phe	Cervical (70); lung, larynx (65)	PA	Bai et al,[70] Sieber et al[71]
LS384	Japanese	Breast: ER/PR+, HER2−	48	None	*MUTYH*	NM_001048171.1:c.892-2A>G	Splice acceptor	Not applicable	Breast (40s); gastric (90); ovarian (40s)	PA	Novel
LS400	NH white	Breast: ER/PR+, HER2+	50	None	*NBN*	NM_002485.4:c.643C>T	Missense	Arg215Trp	Breast (70s); melanoma (49)	PA	Seemanová et al[72]
LS430	NH white	Breast: ER/PR+, HER2−	61	None	*SLX4*	NM_032444.2:c.5229insG	Frameshift	Gln1744AlafsX34	Breast (n = 3; 40s); colon (n = 2, 39, 73); melanoma (27)	PA	Novel
LS443	NH white	No personal cancer history	None	None	*CDH1; NBN*	NM_004360.3:c.532-18C>T; NM_002485.4:c.643C>T	Intronic; Missense	Not applicable; Arg215Trp	Breast (n = 4; 50s-70s); colon (70s); ovary (60s)	PA; PA	Seemanová et al,[72] Suriano et al[73]
LS456	NH white	Breast: ER/PR+, HER2−	49	None	*ATM*	NM_000051.3:c.7271T>G	Missense	Val2424Gly	Breast (n = 2; 40s-60s); colon (n = 2; 30s-40s); melanoma (n = 2; 40, 58)	PA	McConville et al,[74] Mitui et al[75]
LS462	NH white	Breast: ER/PR+, HER2−	42	None	*MUTYH*	NM_001048171.1:c.494A>G	Missense	Tyr165Cys	Breast (56); lung (54, 76); pancreas (44)	PA	Ali et al,[67] Eliason et al[68]

Abbreviations: +, positive; −, negative; DCIS, ductal carcinoma in situ; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; NA, not actionable; NH, non-Hispanic; PA, potentially actionable; PR, progesterone receptor.

*Family history of cancer in first- and second-degree relatives; parenthetical information includes patient age/decade of diagnosis (for multiple affected relatives, the number affected is reported, eg, n = 3; followed by relatives' ages).

concordant with prior testing for 197 of 197 participants, and pathogenicity interpretation was concordant for 196 (99.5%) of 197 participants (95% CI, 96.9% to 100%).

Pathogenic Variants in Other Genes

Sixteen pathogenic variants were detected in women who tested negative for *BRCA1/2* mutations, for a prevalence of 11.4% (95% CI, 7.0% to 17.7%). The affected genes were *ATM* (two women), *BLM* (one woman), *CDH1* (one woman), *CDKN2A* (one woman), *MLH1* (one woman), *MUTYH* (five women), *NBN* (two women), *PRSS1* (one woman), and *SLX4* (two women). Eleven variants were previously reported in the literature, and five were novel. Consistent with the larger study sample and source population, most of the women carrying pathogenic variants (80%) had a personal history of breast cancer; 67% were non-Hispanic white, 20% were non-Hispanic Asian, and 13% were Hispanic (Table 3).

Variants of Uncertain Significance

A total of 428 VUS were identified in 39 genes among 175 participants. Per participant, the average number of VUS across all genes was 2.1 (standard deviation, 1.5; Fig 1A). Per gene, the median number of VUS detected across all 198 participants was eight, ranging from zero (*PTEN*, *SMAD4*, *SPINK1*) to 36 (*APC*; Fig 1B). VUS protein effects were as follows: 49 intronic (11.4%), 269 missense (62.9%), 86 silent (20.1%), eight noncoding (1.9%), two in-frame codon loss (0.5%), one in-frame codon gain (0.2%), and 13 unknown (3%). Most of the VUS were novel (n = 380; 88.8%); the PolyPhen program predicted that 151 were benign (35.3%), 65 were probably damaging (15.2%), and 50 were possibly damaging (11.7%).[76,77]

Clinical Interpretation of Pathogenic Variants

Of 16 pathogenic variants in genes other than *BRCA1/2*, we determined that 15 met our criteria for being potentially actionable (Table 3).[69,78,79] One missense variant in *CDKN2A* (NM_000077.4: c.146T>C) was classified as likely pathogenic based on functional evidence,[80-83] but with conflicting reports[69]; it was therefore considered not actionable.

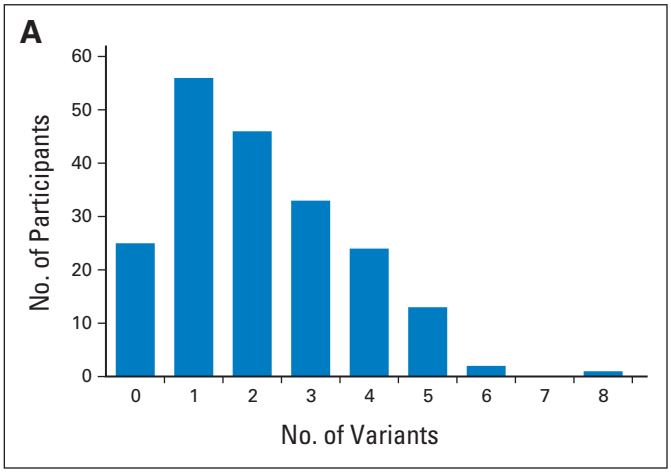

Fig 1. (A) Frequency of variants of uncertain significance, per participant, across 42 sequenced genes.

Participant Notification and Clinical Follow-Up

Given the clinical significance of the pathogenic variants deemed potentially actionable, permission was obtained from the Stanford University IRB to contact participants again and offer them the results of their research testing. A genetic counselor (K.E.K.) attempted to call by telephone the 14 women who carried the 15 potentially actionable variants. Of these 14, three were lost to follow-up. Of the 11 women we were able to reach by telephone, 10 accepted a counseling appointment, and one was deceased but her children accepted an appointment. During counseling appointments, participants reviewed and signed a new IRB-approved informed consent document, confirming their willingness to receive study results. The appointments were led by a genetic counselor (K.E.K.) and an oncologist (A.W.K. or J.M.F.); they included an explanation of the results' estimated contribution to cancer risk, CLIA-approved confirmatory testing, and a discussion of risk-adapted screening or prevention options. Six participants (who carried the *ATM*, *BLM*, *CDH1*, *NBN*, and *SLX4* variants) were advised to consider annual breast MRIs because of an estimated doubling of breast cancer risk,[17,18,23,24,34-36,45,46,59-61,84,85] and six participants (who carried the *CDH1*, *MLH1*, and *MUTYH* variants) were advised to consider frequent colonoscopy and/or endoscopic gastroduodenoscopy (once every 1 to 2 years) due to estimated increases in gastrointestinal cancer risk.[1-4,86] One participant (LS294; Table 3) who had triple-negative breast cancer at age 35 years was found to carry a frameshift *MLH1* mutation consistent with Lynch syndrome. Her breast tumor was analyzed by immunohistochemistry and demonstrated absent *MLH1* and *PMS2* expression. She had previously undergone hysterectomy for endometrial carcinoma at age 46 years. After sequencing revealed she had Lynch syndrome, she underwent risk-reducing salpingo-oophorectomy and early colonoscopy, the latter identifying a tubular adenoma that was excised (Fig 2).

DISCUSSION

We evaluated the clinical performance of a multiple-gene sequencing panel among 198 women meeting evidence-based practice guidelines for *BRCA1/2* testing. We detected 16 pathogenic variants (11.4%; 95% CI, 7.0% to 17.7%) in other genes among women who tested negative for *BRCA1/2* mutations, with 15 variants (10.6%; 95% CI, 6.5% to 16.9%) warranting discussion of more intensive screening or prevention. This is a significant yield of potentially actionable results, comparable to the 5% to 10% probability threshold endorsed by guidelines and payers for *BRCA1/2* and Lynch syndrome testing.[1-3] Up to 10 years after research sample donation, most participants (78.6%) could be reached for results notification, and all accepted confirmatory CLIA-approved testing and counseling. Although further research is required to guide practice, these findings provide an early signal for the clinical relevance of multiple-gene sequencing in cancer-risk assessment.

Multiple-gene sequencing panels have emerged over the last few years, with clinical availability as of 2012.[8,87] However, the United States Supreme Court decision against gene patenting in June 2013 permitted the inclusion of *BRCA1/2* in panels offered by several companies,[87] and a concurrent drop in pricing has provided incentive for the uptake of these new products because a six-gene panel now costs no more than a two-gene test. Owing to absent data on clinical performance, expert opinion statements urge caution in using multiple-gene panels outside a clinical trial.[2,8] Our current study was nested

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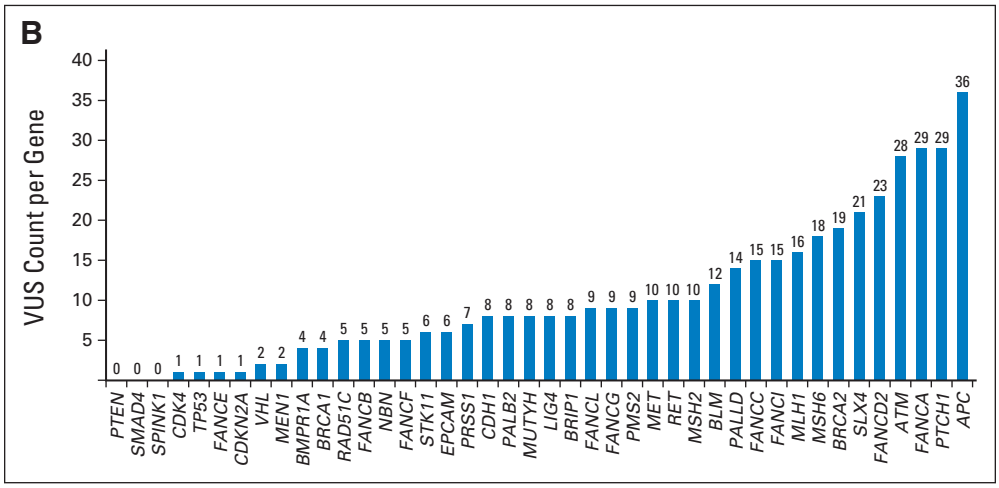

Fig 1. (B) Variants of uncertain significance (VUS) count, per gene, across 198 participants.

within a Clinical Cancer Genetics practice adherent to evidence-based testing guidelines,[2] and its findings should generalize broadly across clinical settings. Furthermore, its results address real-world challenges of multiple-gene sequencing. Our definition of pathogenic variants as potentially actionable generally follows recommendations of the American College of Medical Genetics (for reporting of incidental findings, although some genes we reported fall outside of these recommendations)[58] and other guideline organizations (for breast MRI screening with a two-fold relative risk increase, which we estimated using published literature on mutation penetrance).[59-61] Notably, there are no data as yet on the risk-benefit ratio of breast MRI screening among patients with pathogenic variants in genes of moderate penetrance (eg, *ATM*, *BLM*). Given the remaining uncertainty in penetrance estimates for such variants, we cannot precisely estimate their contribution to a woman's age-specific cancer risks and, therefore, the optimal breast screening protocol and age of initiation remain unknown. A subjective component is unavoidable in interpreting unfamiliar variants[11]; other clinicians might make different judgements about patient notification and management. Nonetheless, our experience of results disclosure and risk-adapted intervention may inform future applications of multiple-gene sequencing.

Some of the pathogenic variants we identified would be detected by adherence to current practice guidelines. For example, the participant (LS294) who was found to carry an *MLH1* mutation would now receive Lynch syndrome testing because she had endometrial carcinoma at age 46 years.[1] When she was clinically assessed several years ago, however, Lynch syndrome testing was not routine for early endometrial cancer, and it is still underutilized.[88,89] Moreover, this patient's early triple-negative breast cancer would place *BRCA1/2* testing first in most clinicians' differential diagnoses,[2] such that sequential single-syndrome testing would be slower to provide the correct answer than a multiplex approach. In contrast, other pathogenic variants lack such clear

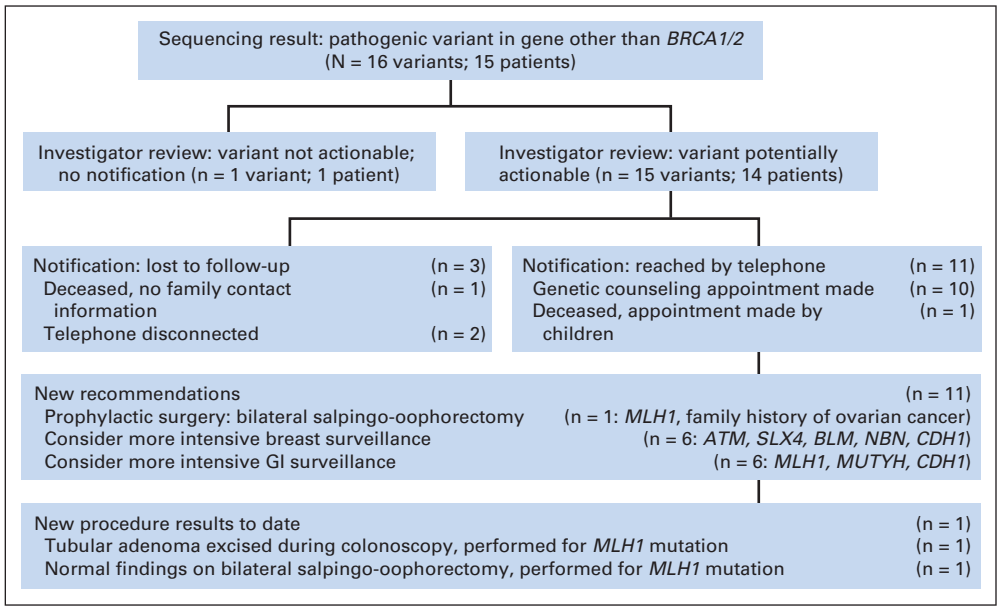

Fig 2. Pathogenic variant interpretation, participant notification, and clinical follow-up.

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guidelines and present significant challenges. One example is the frameshift *SLX4* mutations identified in participants LS347 and LS430. Recently identified as causing Fanconi's Anemia, *SLX4* mutations are predicted to convey a two-fold increase in breast cancer risk that is similar to other pathway components such as *PALB2*. Though some publications support this risk association, *SLX4* mutations appear rare among breast cancer families.[45,46,84] Participants LS347 and LS430 each had a striking family history that included at least three breast cancers diagnosed before age 50 years; we therefore recommended breast MRI screenings, consistent with guidelines for women with increased risk.[59-61] As another example, germline *CDH1* mutations convey substantial risks of gastric and breast cancer in hereditary gastric cancer families[90]; the inadequacy of endoscopic screening leaves prophylactic gastrectomy as the only effective intervention.[6] However, it is unknown whether patients who were found to carry *CDH1* mutations incidentally, without family gastric cancer history, should undergo such life-changing surgery. Because participant LS443's *CDH1* variant has not been reported among gastric cancer patients, we recommended close surveillance rather than gastrectomy. These ambiguous cases illustrate the complexities of multiple-gene sequencing in clinical practice and the potential hazards of unwarranted intervention. As a conservative approach to identified pathogenic variants of uncertain penetrance, we discussed increasing the frequency and/or intensity of cancer screening (Fig 2), but we judged the evidence insufficient to support recommendation of irreversible, invasive interventions such as prophylactic surgery. Another crucial unanswered question is whether testing negative for an identified familial mutation in genes of moderate penetrance (eg, *PALB2*, *CHEK2*) implies that a patient's risk of developing mutation-associated cancers is no greater than that of the general population; we require this missing information to enable risk-appropriate screening. Population-based studies of mutation penetrance and clinical trials of risk-reducing interventions are urgently needed to guide such difficult clinical decisions.

A major concern is the discovery of variants of uncertain significance, which do not contribute to risk assessment and may prompt anxiety and overtreatment. With widespread *BRCA1/2* testing, the prevalence of VUS has declined to an acceptably low rate of 2% to 5%.[91] Predictably, we found that sequencing 42 genes identified VUS in many participants (88%), averaging 2.1 VUS per participant. Our demonstration of this anticipated finding (that sequencing more genes yields more VUS, most of them novel) speaks to nascent efforts toward clinical whole-genome analysis. Moreover, it raises the question of a break-even point: what is the best set and number of genes to maximize information and minimize noise? Consistent with our IRB-approved protocol, we elected not to recontact about VUS, because these results lack practical implications. Outside a study, however, such a decision might conflict with patient autonomy, and the current standard of clinical VUS reporting will likely pertain.[2] Therefore, it is crucial to develop methods for reclassifying VUS quickly and to communicate evolving VUS interpretations according to patients' preferences and understanding. This priority underscores the critical importance of sharing open databases of VUS identified through various genetic sequencing efforts, public and private.

Our study has limitations. The 42 genes that we selected reflect published literature but an optimal multiple-gene panel for routine diagnostic use remains to be defined. We conducted this study in an academic center, within a specialized Clinical Cancer Genetics service that is not universally available. Although participants did not differ significantly from the source population on most measured criteria, it is possible that they had subtle features of personal or family history that suggested greater inherited risk, which might have increased their willingness to donate a research blood sample. Because they were clinically accrued, participants do not represent the entire United States population. Another limitation is the absence of patient-reported preferences and outcomes, which will be a critical consideration in translating next-generation sequencing into practice. Our current results may inform the design of studies that address these important unanswered questions.

To our knowledge, this is the first clinical evaluation of next-generation sequencing among patients referred for breast and ovarian cancer risk assessment. Undoubtedly, multiple-gene sequencing raises many questions about results interpretation and patient counseling. Our study demonstrates an early signal for the clinical relevance of multiple-gene sequencing and provides a strong rationale for future research to define its most effective use.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) and/or an author's immediate family member(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.
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GLOSSARY TERMS

BRCA1: a tumor suppressor gene known to play a role in repairing DNA breaks. Mutations in this gene are associated with increased risks of developing breast or ovarian cancer.

BRCA2: a tumor suppressor gene whose protein product is involved in repairing chromosomal damage. Although structurally different from BRCA1, BRCA2 has cellular functions similar to BRCA1. BRCA2 binds to RAD51 to fix DNA breaks caused by irradiation and other environmental agents. Also known as the breast cancer 2 early onset gene.

CDH1: cadherin 1 gene. Mutations in this gene are correlated with gastric, breast, colorectal, thyroid, and ovarian cancers.

Lynch syndrome: hereditary nonpolyposis colorectal cancer (HNPCC). A cancer syndrome characterized by Henry T. Lynch in 1966, this genetic condition has a high risk of colon cancer as well as other cancers including endometrial, ovary, stomach, small intestine, hepatobiliary tract, upper urinary tract, brain, and skin.

MLH1 (MutL homolog 1): a DNA mismatch repair enzyme. MLH1 is responsible for overall fidelity of DNA replication.

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