Detection of inherited mutations for breast and ovarian cancer using genomic capture and massively parallel sequencing

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Inherited loss-of-function mutations in the tumor suppressor genes *BRCA1*, *BRCA2*, and multiple other genes predispose to high risks of breast and/or ovarian cancer. Cancer-associated inherited mutations in these genes are collectively quite common, but individually rare or even private. Genetic testing for *BRCA1* and *BRCA2* mutations has become an integral part of clinical practice, but testing is generally limited to these two genes and to women with severe family histories of breast or ovarian cancer. To determine whether massively parallel, "next-generation" sequencing would enable accurate, thorough, and cost-effective identification of inherited mutations for breast and ovarian cancer, we developed a genomic assay to capture, sequence, and detect all mutations in 21 genes, including *BRCA1* and *BRCA2*, with inherited mutations that predispose to breast or ovarian cancer. Constitutional genomic DNA from subjects with known inherited mutations, ranging in size from 1 to >100,000 bp, was hybridized to custom oligonucleotides and then sequenced using a genome analyzer. Analysis was carried out blind to the mutation in each sample. Average coverage was >1200 reads per base pair. After filtering sequences for quality and number of reads, all single-nucleotide substitutions, small insertion and deletion mutations, and large genomic duplications and deletions were detected. There were zero false-positive calls of nonsense mutations, frameshift mutations, or genomic rearrangements for any gene in any of the test samples. This approach enables widespread genetic testing and personalized risk assessment for breast and ovarian cancer.

BRCA1 | BRCA2 | genomics | next-generation sequencing | genetic testing

Inherited mutations in *BRCA1* and *BRCA2* predispose to high risks of breast and ovarian cancer. Lifetime risks of breast cancer are as high as 80% among women with mutations in these genes, and lifetime risks of ovarian cancer are greater than 40% for carriers of *BRCA1* mutations and greater than 20% for carriers of *BRCA2* mutations (1). Inherited mutations in the Fanconi anemia genes *BRIP1* (*FANCJ*) and *PALB2* (*FANCN*) are associated with 20–50% lifetime risks of breast cancer (2, 3). Inherited mutations in *TP53*, *PTEN*, *STK11*, and *CDH1* are associated with moderate to very high risks of breast cancer in the context of Li-Fraumeni syndrome, Cowden syndrome, Peutz-Jeughers syndrome, and hereditary diffuse gastric cancer syndrome, respectively (4, 5, 6, 7). Inherited mutations in several of the genes responsible for hereditary nonpolyposis colon cancer and endometrial cancer are also associated with elevated risks of ovarian cancer (8).

Genetic testing for *BRCA1* and *BRCA2* mutations has become an integral part of clinical practice for women with severe family histories of breast or ovarian cancer, whether newly diagnosed or still clinically asymptomatic. However, as many as 50% of breast cancer patients with inherited mutations in *BRCA1* and *BRCA2* do not have close relatives with breast or ovarian cancer because their mutation is paternally inherited, the family is small, and by chance no sisters or paternal aunts have inherited the mutation of the family (1). Women in such families who carry *BRCA1* or *BRCA2* mutations have the same high risks of breast and ovarian cancer as women from high-incidence families. At present, women from such families rarely use genetic services.

In the United States, genetic testing of *BRCA1* and *BRCA2* is carried out almost exclusively by a single commercial company, whose protocol is based on PCR amplification of individual exons and Sanger sequencing of the products (9). In 2007, a quantitative DNA measurement assay (BART) was added as a supplementary test to detect large exonic deletions and duplications that are not detectable by PCR amplification approaches (BRACAnalysis Technical Specifications (updated February 2009) http://www.myriadtests.com/provider/doc/BRACAnalysis-Technical-Specifications.pdf). In Europe, genetic testing of BRCA1 and BRCA2 is more widely available (10, 11). Sequencing of the more moderate-risk breast cancer genes is available in various research or commercial diagnostic laboratories (GeneClinics http://www.ncbi.nlm.nih.gov/sites/GeneTests/?db=GeneTests), but is not routinely performed.

Recent advances in sequencing technologies have dramatically increased the speed and efficiency of DNA testing (12–16). Medical screening of genes responsible for disease generally requires an enrichment step before sequencing (17). This enrichment improves accuracy of mutation detection and reduces cost per sequenced nucleotide. To identify as many mutations as possible that are responsible for inherited predisposition to breast and ovarian cancer, it is useful to analyze multiple genes, not only *BRCA1* and *BRCA2*. The mutational spectra of these genes include single-nucleotide variants, small insertions and deletions, and large genomic rearrangements spanning multiple kilobases. An approach to mutation detection based on next-generation sequencing must be able to accurately and cost-effectively detect all these classes of mutations before it can be used in a clinical diagnostic setting. This project was proof of principle for the application of solution capture and next-generation sequencing to mutation detection for patients at high risk of breast or ovarian cancer.

Results

Our goal was to evaluate the accuracy of DNA capture followed by massive parallel sequencing for identification of inherited mutations in breast and ovarian cancer genes. To carry out DNA capture, we designed oligonucleotides to target complete genomic sequence of 21 genes responsible for inherited risk of these cancers (Table 1). Oligonucleotides were designed to cover coding regions, noncoding intronic sequences, and 10-kb genomic sequence flanking each gene. After repetitive DNA elements were masked, total DNA targeted was ≈1 megabase.

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Table 1. Genomic regions targeted for breast and ovarian cancer genes

Gene	Chromosome	Captured genomic region	
		Start	End
BRCA1	17	41,186,313	41,347,712
BRCA2	13	32,879,617	32,983,809
CHEK2	22	29,073,731	29,147,822
PALB2	16	23,604,483	23,662,678
BRIP1	17	59,759,985	59,940,755
p53	17	7,561,720	7,600,863
PTEN	10	89,613,195	89,738,532
STK11	19	1,195,798	1,238,434
CDH1	16	68,761,195	68,879,444
ATM	11	108,083,559	108,249,826
BARD1	2	215,583,275	215,684,428
MLH1	3	37,024,979	37,102,337
MRE11	11	94,140,467	94,237,040
MSH2	2	47,620,263	47,720,360
MSH6	2	48,000,221	48,044,092
MUTYH	1	45,784,914	45,816,142
NBN	8	90,935,565	91,006,899
PMS1	2	190,638,811	190,752,355
PMS2	7	6,002,870	6,058,737
RAD50	5	131,882,630	131,989,595
RAD51C	17	56,759,963	56,821,692

Fig. 1. Screening for mutations in breast and ovarian cancer genes using targeted DNA capture and next-generation sequencing.

The mutation screening process is outlined in Fig. 1. DNA was extracted from blood and sonicated, and libraries were prepared with a mean insert size of 200 bp. Libraries were hybridized in solution to the custom oligonucleotides and then sequenced on an Illumina Genome Analyzer IIX to generate 2- × 76-bp paired-end reads. An average of 2.4 gigabase (Gb) per sample (range 1.8–4.4 Gb per sample) of high-quality sequence was obtained at targeted sites, representing an average 1,286-fold coverage per nucleotide (range across all samples was 781- to 1854-fold average coverage per nucleotide). DNA sequences were aligned to the human reference genome. Nucleotide coordinates of rare variants were identified as described in *Materials and Methods*. We required that a potential variant be present on both sequenced DNA strands and represent ≥15% of total reads at that site to be further evaluated. The 15% threshold was chosen because the mutation *CHEK2_1100delC* was present on only 15% of reads at

this site (Table 2) as the result of the existence of *CHEK2* pseudogenes on chromosomes 15 and 16. Common polymorphisms were excluded by comparison with dbSNP130. However, because dbSNP erroneously includes some severe disease-associated mutations as benign polymorphisms (e.g., *p53_721G > A*) (18), we also compared all variants to gene-specific mutation databases. Candidate variants were categorized by gene location (intergenic, intronic, or exonic) and by predicted effect (frameshift, in-frame insertion or deletion, synonymous substitution, nonsynonymous substitution, splice site alteration, or nonsense). Each candidate variant was evaluated by conventional Sanger sequencing. All samples were tested blind to their mutation.

Table 2. Point mutations and small insertions and deletions identified by the assay

Gene	Nucleotide	Effect	Type	Size (bp)	Mutant sites identified			No. of reads		
					Chromosome	Start	End	Wild type	Variant	% Variant
BRCA1	4510 del3ins2	1465 stop	Deletion-insertion	1	17	41,228,596	41,228,597	525	596	0.53
BRCA1	5083 del19	1657 stop	Deletion	19	17	41,222,949	41,222,968	700	644	0.48
BRCA1	5382 insC	1829 stop	Insertion	1	17	41,209,080	41,209,081	606	596	0.50
BRCA2	999 del5	273 stop	Deletion	5	13	32,905,141	32,905,146	363	229	0.39
BRCA2	1983 del5	585 stop	Deletion	5	13	32,907,366	32,907,371	304	258	0.46
BRCA2	6174 delT	2003 stop	Deletion	1	13	32,914,438	32,914,439	565	661	0.54
BRCA2	9179 C > G	2984 stop	Nonsense	1	13	32,953,650		391	361	0.48
BRIP1	3401 delC	1149 stop	Deletion	1	17	59,761,006	59,761,007	651	486	0.43
CDH1	591 G > A	157 stop	Nonsense	1	16	68,842,406		421	359	0.46
CHEK2	1100 delC	381 stop	Deletion	1	22	29,091,857	29,091,858	3,293	586	0.15
MLH1	ivs14(-1) G > A	568 stop	Splice	1	3	37,083,758		1,024	683	0.40
MSH2	1677 T > A	537 stop	Nonsense	1	2	47,693,895		575	552	0.49
p53	721 G > A	R175H	Missense	1	17	7,578,406		449	306	0.41
PALB2	509 delGA	183 stop	Deletion	2	16	23,647,357	23,647,359	1,283	1,233	0.49
STK11	ivs6(-1) G > A	316 stop	Splice	1	19	1,221,947		722	572	0.44

Gene	Genomic event	Mutant sites identified by assay				
		Chromosome	Start*	End*	Size (bp)	Ratio†
BRCA1	Deletion exons 1–15	17	41,226,145	41,327,157	101,013	0.509
BRCA1	Duplication exon 13	17	41,230,562	41,235,836	5,275	1.578
BRCA1	Deletion exons 14–20	17	41,203,975	41,229,297	25,323	0.519
BRCA1	Deletion exon 17	17	41,219,596	41,219,755	160	0.495
BRCA2	Deletion exons 1–2	13	32,889,020	32,890,900	1,881	0.489
BRCA2	Deletion exon 21	13	32,950,734	32,952,070	1,337	0.544

*Breakpoints are flanked by *Alu* and other repeats, which are not captured.
†Reads per base pair for deletion or duplication/reads per base pair for wild-type genotype.

All mutations in the test series were accurately identified and there were zero false-positive calls of mutations in any gene in any of the samples. Point mutations and small insertions and deletions in *BRCA1*, *BRCA2*, *BRIP1*, *CDH1*, *CHEK2*, *MLH1*, *MSH2*, *p53*, *PALB2*, and *STK11* ranged in size from 1 to 19 bp (Table 2). The genomic base pairs of each were correctly identified. In addition, by comparing the number of sequence reads at each base pair for each sample to the number of reads at the same base pair for all other samples in the experiment, we screened for large deletions and duplications at each of the 21 loci. Deviations from diploidy were defined as sites at which a test sample yielded <60% or >140% the average number of reads of the other samples in the experiment. We accurately identified the five genomic deletions and one genomic duplication (Table 3, Fig. 2), determining breakpoints on the targeted sequence within 1 kb. Each large deletion and duplication is flanked by *Alu* sequences that mediate the mutation. Because *Alu* repeats are not targeted by the oligonucleotides in the capture pool, the exact breakpoints within flanking Alu repeats are not determinable. There was complete concordance between deletions and duplications identified by our read-depth algorithm and by the multiple ligation probe assay (19).

Discussion

The landscape of genetic testing in the United States was changed on March 29, 2010, by the decision of Judge Robert Sweet of the Federal District Court in Manhattan, which invalidated Myriad Genetics' patents on the *BRCA1* and *BRCA2* genes (20). By declaring that genes are products of nature and therefore not subject to patent, he called into question patents filed on thousands of human genes. In this context, it may be that tools for more efficient genetic testing for cancer susceptibility genes will be developed and clinically applied.

The availability of more rapid and cost-effective testing for multiple breast and ovarian cancer susceptibility genes has major clinical implications. *BRCA1* and *BRCA2* are the genes most commonly implicated in hereditary breast or ovarian cancer and were patented by Myriad Genetics and the University of Utah after their identification in 1994 and 1995, respectively. Since then, Myriad Genetics has been the sole source in the United States for commercial testing for inherited mutations in *BRCA1* and *BRCA2*. The standard test consists of DNA sequencing of both genes and screening for five large deletions and duplications in *BRCA1* and costs $3,340. Comprehensive testing for gene rearrangements is offered as a separate test by Myriad Genetics at an additional cost of $650. If *BRCA1* and *BRCA2* testing is negative (i.e., wild type), testing for other breast or ovarian cancer genes is typically done selectively, when mutations in certain other genes are suspected on the basis of family history, personal history, or findings from physical examination (21). Sequencing additional genes can add thousands more dollars to the cost of genetic testing. It is possible to identify mutations in the 21 known breast and ovarian cancer genes in

one sample for a cost of reagents and consumables less than $1,500. Given the massive redundancy of read depth that we achieved by running a single sample in one flow cell lane, it is likely that a barcoding or indexing strategy would be feasible and reduce the cost to less than $500 per sample.

Other applications of next-generation sequencing to genetic testing of *BRCA1* and *BRCA2* have been suggested (22–24). The approach described here differs in that we evaluated multiple genes, in addition to BRCA1 and BRCA2, and we evaluated all classes of mutation. Our next-generation sequencing approach identified a wide range of mutations in a variety of genes in 100% of our test cases with zero spurious mutations called. Target mutations included single base substitutions and deletions and insertions of varying sizes. Importantly, we easily identified six large deletions and duplications, all of which would have been
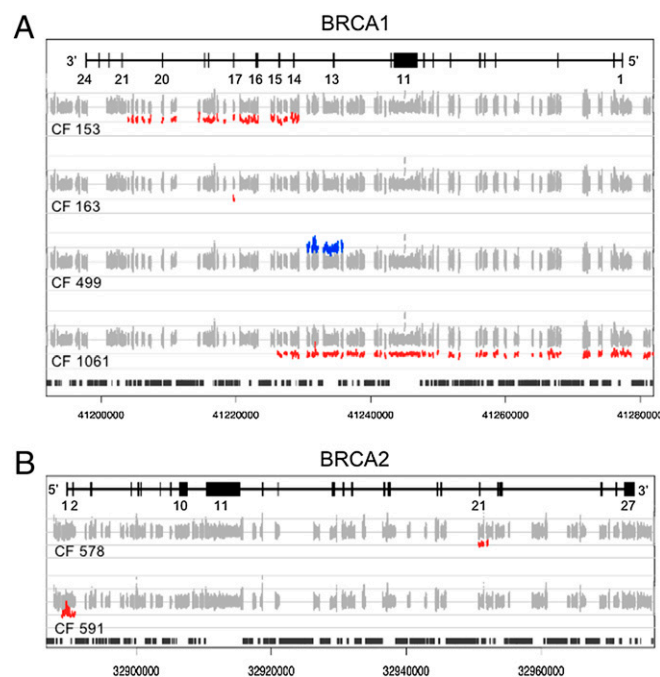
Fig. 2. Large genomic deletions and duplications in *BRCA1* and *BRCA2* identified by analysis of the read depth of sequencing data. Normalized numbers of sequencing reads are indicated for each gene. Exons are indicated by black vertical lines and intervening introns by horizontal lines. Numbers of reads for each base pair are represented in gray. Numbers of reads for each sample deviating from the median of all samples for that base pair occur at deletions (red) and at duplications (blue). DNA repetitive elements are indicated by black vertical bars (bottom of *A* and *B*). (*A*) The *BRCA1* locus in DNA from families 153, 163, 499, and 1061. (*B*) The *BRCA2* locus in DNA from families 578 and 591.

missed by standard sequencing (19). This approach thus obviates the need for separate testing for gene rearrangements after Sanger DNA sequencing (25).

Because testing for mutations in genes other than *BRCA1* and *BRCA2* is done only selectively, the proportion of breast and ovarian cancers attributable to inherited mutations in these other genes is not known. By allowing comprehensive parallel testing of multiple cancer susceptibility genes, we will be able to confidently identify the fraction of women with breast or ovarian cancer who carry a germline alteration in a cancer susceptibility allele and the characteristics of the tumors of patients' inherited mutations in various genes.

The cost savings in applying such technologies will allow the application of genetic testing to a wider range of individuals than is the current standard. Presently, patients who have a relatively high pretest probability of having a mutation in a given cancer susceptibility allele are chosen for testing (26, 27). However, many breast and ovarian cancer patients with *BRCA1* or *BRCA2* mutations have a negative family history for cancer (1). Since the advent of inhibitors of the Poly (ADP ribose) polymerase (PARP) enzyme, which effectively kill *BRCA1*- and *BRCA2*-mutated carcinomas, understanding the genetic basis of human cancers has therapeutic as well as preventive implications (28, 29). The availability of PARP inhibitors has increased the clinical incentive to identify *BRCA1* and *BRCA2* mutations in women with breast or ovarian cancer.

Being able to test for multiple cancer susceptibility genes simultaneously will add complexity to the clinical interpretation of results. More variants of uncertain significance will be identified, and clinical recommendations may not be standardized for mutations in some cancer susceptibility genes. As the complexity of genetic testing for cancer risk increases, we emphasize the importance of including a medical geneticist or certified genetic counselor in the testing process. Consequently, these types of tests may not be appropriate to order in a primary practice setting or directly from the company as a product of direct-to-consumer marketing.

As more next-generation sequencing technologies become available for genetic testing, results on sensitivity and specificity should be made freely accessible to those who order the test. Hopefully, comparisons of various technologies will also become available. Guidelines for application of next-generation sequencing to genetic diagnostics are currently under development (30). At present, we believe that apparently positive tests should be validated by standard Sanger sequencing of the patient's DNA before results are reported to the patient. Sanger sequencing would verify the mutation and provide the basis for a simplified test for at-risk relatives. For mutation detection, our goal was to extend genetic testing for inherited risk of breast and ovarian cancer to more women by providing an approach that offers substantially lower cost while maintaining very high sensitivity and specificity.

Materials and Methods

Study Subjects. Participants were 20 women diagnosed with breast or ovarian cancer and with a known mutation in one of the genes responsible for inherited predisposition to these diseases. The critical mutation for each patient had been previously identified by Sanger sequencing of PCR amplicons or by multiplex ligation-dependant probe amplification, as described (19). The study was approved by the institutional review board at the University of Washington. All participants provided informed consent.

Capture and Sequencing of Genomic DNA. Genomic DNA was captured by hybridization in solution to custom-designed cRNA oligonucleotide baits (31) following the manufacturer's protocols (Agilent Technologies). BED files of genomic locations of all cRNA oligonucleotide probes are freely available on request to the authors. Captured library DNA (9 pM) was denatured and subjected to cluster amplification on a Paired End Flow Cell v4 with a cBot instrument (Illumina) to generate raw cluster intensity of ~700,000 mm^2. Sequencing was performed on a Genome Analyzer GAIIX for 2×76 cycles using Cycle Sequencing v4 reagents (Illumina).

Average coverage for the captured regions ranged from 781- to 1,854-fold per site. To determine if coverage was substantially lower for any region, we calculated the proportion of base pairs for each locus that were captured by <100 reads. All regions were covered by >20 reads. Proportions of base pairs covered by <100 reads were 0.082 for *PMS2*, 0.067 for *STK11*, 0.023 for *PTEN*, and 0.002 for *MRE11* and *MUTY*. Content of guanine and cytosine (GC) basepairs at these poorly covered regions was high; most were CpG islands. Such regions are also refractive to PCR-based sequencing methods. All base pairs of *BRCA1*, *BRCA2*, *CHEK2*, *PALB2*, *BRIP1*, *p53*, *CDH1*, *ATM*, *BARD1*, *MLH1*, *MSH2*, *NBN*, *PMS1*, *RAD50*, and *RAD51C* were captured by >100 reads.

Bioinformatic Analysis of DNA Variants. Sequencing data were processed through Illumina pipeline v1.6 using default parameters. Reads of high quality were mapped to the reference human genome sequence (GRCh37, UCSC hg19) using MAQ 0.7.1 with default parameters (32). Reads outside the targeted sequences were discarded and statistics on coverage were collected from the remaining reads. Across samples evaluated with this oligonucleotide pool, an average of 69% of reads were on target. Potential single-base-pair variants and small insertions and deletions were identified using the MAQ Perl-based filter after alignment (-map), assembly (-assembly), and consensus calling (-cns2snp) with default parameters.

To detect large genomic deletion and duplication mutations, we developed a script to count sequence reads on captured DNA and then converted total read depth at a given genomic location into deletion and duplication calls. Read depth was corrected for oligonucleotide probe coverage and local GC content. After testing different window sizes, we found that a 100-bp window gave the strongest relationship between GC content and base coverage. We normalized to median coverage across all samples using invariant set methods (33) and then compared each individual's coverage to the median coverage. The derived coverage ratio was then subjected to our sliding-window deletion and duplication-calling method. The minimum accepted call length for deletions and duplications was 100 bp, with threshold ratios <0.6 for deletions and >1.4 for duplications. Using these methods, we identified all six known deletions and duplications. We compared the difference between the number of reads for the sample carrying the putative deletion or duplication with the median number of reads at the corresponding base pair for all samples. Differences were compared by *t* tests for each event defined by this method. For all deletions and duplications, differences in read depth were significant at $P < 10^{-10}$.

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