Spectrum of Mutations in *BRCA1*, *BRCA2*, *CHEK2*, and *TP53* in Families at High Risk of Breast Cancer

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Context Genetic testing for inherited mutations in *BRCA1* and *BRCA2* has become integral to the care of women with a severe family history of breast or ovarian cancer, but an unknown number of patients receive negative (ie, wild-type) results when they actually carry a pathogenic *BRCA1* or *BRCA2* mutation. Furthermore, other breast cancer genes generally are not evaluated.

Objective To determine the frequency and types of undetected cancer-predisposing mutations in *BRCA1*, *BRCA2*, *CHEK2*, *TP53*, and *PTEN* among patients with breast cancer from high-risk families with negative (wild-type) genetic test results for *BRCA1* and *BRCA2*.

Design, Setting, and Participants Between 2002-2005, probands from 300 US families with 4 or more cases of breast or ovarian cancer but with negative (wild-type) commercial genetic test results for *BRCA1* and *BRCA2* were screened by multiple DNA-based and RNA-based methods to detect genomic rearrangements in *BRCA1* and *BRCA2* and germline mutations of all classes in *CHEK2*, *TP53*, and *PTEN*.

Main Outcome Measures Previously undetected germline mutations in *BRCA1*, *BRCA2*, *CHEK2*, *TP53*, and *PTEN* that predispose to breast cancer; frequencies of these mutations among families with negative genetic test results.

Results Of the 300 probands, 52 (17%) carried previously undetected mutations, including 35 (12%) with genomic rearrangements of *BRCA1* or *BRCA2*, 14 (5%) with *CHEK2* mutations, and 3 (1%) with *TP53* mutations. At *BRCA1* and *BRCA2*, 22 different genomic rearrangements were found, of sizes less than 1 kb to greater than 170 kb; of these, 14 were not previously described and all were individually rare. At *CHEK2*, a novel 5.6-kb genomic deletion was discovered in 2 families of Czechoslovakian ancestry. This deletion was found in 8 of 631 (1.3%) patients with breast cancer and in none of 367 healthy controls in the Czech and Slovak Republics. For all rearrangements, exact genomic breakpoints were determined and diagnostic primers validated. The 3 families with *TP53* mutations included cases of childhood sarcoma or brain tumors in addition to multiple cases of breast cancer.

Conclusions The mutational spectra of *BRCA1* and *BRCA2* include many high-penetrance, individually rare genomic rearrangements. Among patients with breast cancer and severe family histories of cancer who test negative (wild type) for *BRCA1* and *BRCA2*, approximately 12% can be expected to carry a large genomic deletion or duplication in one of these genes, and approximately 5% can be expected to carry a mutation in *CHEK2* or *TP53*. Effective methods for identifying these mutations should be made available to women at high risk.

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INHERITED MUTATIONS IN *BRCA1* and *BRCA2* predispose to high risks of breast and ovarian cancer.[1,2] Lifetime risks of breast cancer are as high as 80% among US women with mutations in these genes, and lifetime risks of ovarian cancer are greater than 40% for carriers of the *BRCA1* mutation and greater than 20% for carriers of the *BRCA2* mutation. Risks for young women with inherited *BRCA1* or *BRCA2* mutations are particularly increased.[1] Among white women in the United States, 5% to 10% of breast cancer cases and 10% to 15% of ovarian cancer cases are due to inherited mutations in *BRCA1* and *BRCA2*.[3-10]

Inherited mutations in other genes also influence risk of breast cancer. Inherited deleterious mutations of the cell cycle regulator *CHEK2* are associated with a 2-fold increase in breast cancer risk.[11-13] Inherited mutations of *TP53* in families with Li-Fraumeni syndrome and of *PTEN* in families with Cowden

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syndrome are very rare but when they occur are associated with high risks of early onset breast cancer.[14-18]

Clinical options for women at high genetic risk of breast cancer include screening starting at a young age,[19] the use of highly sensitive detection methods,[20] and prophylactic surgeries of the ovaries or breast.[21-27] Because prophylactic surgeries, while highly effective in reducing risk, are also highly invasive, it is particularly important to distinguish mutation carriers from noncarriers with similarly severe family histories. Women with *BRCA1* or *BRCA2* mutations are possible candidates for such surgeries, whereas women in the same families who by chance carry wild-type alleles are at no increased risk. Molecular testing is the only way to distinguish these alternatives.

Genetic testing to identify deleterious *BRCA1* and *BRCA2* mutations in as-yet unaffected women with severe family histories of breast or ovarian cancer has become an integral part of clinical practice in many communities. Mutation screening of *BRCA1* and *BRCA2* is technically challenging, because each gene harbors more than 1000 different disease-associated mutations, the vast majority of which are individually rare. Therefore, with the exception of identification of founder alleles such as those in the Ashkenazi Jewish population, mutation detection in *BRCA1* and *BRCA2* requires that each gene be screened in its entirety.

In the United States, genetic testing of *BRCA1* and *BRCA2* is carried out almost exclusively by a single commercial firm (Myriad Genetics Inc, Salt Lake City, Utah), whose protocol is to sequence the exons and flanking regulatory regions of each gene and, since 2001, to test for 5 specific larger mutations in *BRCA1*.[28,29] Since commercial screening began 10 years ago, many reports have appeared in the research literature of *BRCA1* and *BRCA2* mutations that were not detected by this approach.[30-36] These mutations went undetected not because of any technical error in commercial testing but because many mutations are inherently not detectable by short-range polymerase chain reaction (PCR) followed by genomic sequencing. To provide accurate and complete information to high-risk patients, it is critical to understand the implications of a negative test result.

Our goal in this study was to determine the frequency and types of undetected cancer-predisposing mutations in *BRCA1*, *BRCA2*, *CHEK2*, *TP53*, and *PTEN* among patients with breast cancer from high-risk families with negative results from commercial genetic testing of *BRCA1* and *BRCA2*. We evaluated DNA and RNA samples from 300 breast cancer probands from high-risk families who had tested negative (ie, wild type) by conventional *BRCA1* and *BRCA2* testing. We used multiple different screening approaches to identify mutations of all genomic classes in *BRCA1*, *BRCA2*, *CHEK2*, *TP53*, and *PTEN*. We sought to define the full range of inherited mutations in these genes predisposing to breast and ovarian cancer and to suggest a more comprehensive genetic screening strategy.

METHODS

Patient Recruitment

Probands of US high-risk families were referred by physicians or genetic counselors following negative (ie, wild-type) genetic test results for mutations in *BRCA1* and *BRCA2*, based on complete sequencing of both genes. Most probands were tested also for the presence of 5 large *BRCA1* mutations included since 2001 in the commercial screening panel. To be eligible for the study, probands must have been diagnosed with invasive breast cancer (at any age) and be a member of a family with at least 4 cases of female breast cancer, ovarian cancer, and/or male breast cancer. Probands were from any locale in the United States and of any self-defined ancestry. Additional informative family members were enrolled whenever possible.

Patients in Czech and Slovak hospital-based series were evaluated only for the 5.6-kb *CHEK2* genomic deletion identified in the US cohort described above. Czech and Slovak patients with breast cancer were identified from Brno and Prague in the Czech Republic and from Bratislava in the Slovak Republic.[37,38] These patients were not selected for family history, but the series was enriched for familial cancer because some patients had been referred previously for genetic testing. Patients known to carry pathogenic mutations in *BRCA1* and *BRCA2* were excluded.

The study was approved by the human subjects review committees of the University of Washington Office of Research (protocol 95-1216), the National Institute of Public Health of the Czech Republic, the Masaryk Memorial Cancer Institute of the Czech Republic, and the Cancer Research Institute of the Slovak Academy of Sciences. All participants provided written informed consent.

DNA Sequencing

BRCA1, *BRCA2*, and *CHEK2* exons and flanking intronic splice sites were evaluated in genomic DNA from probands by conventional DNA sequencing, as previously described.[13,39] The recently discovered *BRCA1* exon 13A was sequenced in 134 of the probands.[40] For families with clinical indications of Li-Fraumeni or Cowden syndromes, *TP53* and *PTEN* were fully sequenced. Primer sequences are available from the authors on request.

Analysis of Variation in Transcript Lengths

Two classes of mutations that would be missed by conventional sequencing are splicing errors due either to genomic rearrangements or to intronic point mutations. One proposed mechanism for such errors is "exonization" of *Alu* sequences, which can result from genomic mutations near *Alu* repeats that create new splice sites, leading to splicing of *Alu* repeats into message and hence to premature stops.[41,42] Either of these mechanisms would lead to transcripts of abnormal lengths. To detect such events, we isolated total RNA from Epstein-Barr virus–transformed lymphoblastoid cell lines using the RNeasy kit (Qiagen, Venlo, the Netherlands) and complementary DNA (cDNA) gen-

erated by random hexamer priming using Superscript II reverse transcriptase (Invitrogen Corp, Carlsbad, Calif). The cDNA was amplified with primer pairs (available from the authors on request) spanning exons 1-11 and 11-24 of *BRCA1*; exons 1-10, 11-18, and 16-27 of *BRCA2*; and exons 1-15 of *CHEK2*. Products of cDNA were electrophoresed on 0.8% agarose gels. Products of PCR that were of aberrant size were gel extracted using QIAquick (Qiagen) and sequenced in both directions. Because this approach will detect both naturally occurring splice isoforms and splicing errors, we sequenced abnormal cDNA products and their genomic counterparts using long-range PCR, as described below. Analysis of cDNA successfully identified multiple mutant messages containing premature stop codons in *BRCA1*, *BRCA2*, and *CHEK2*, all of which proved due to genomic rearrangements. Detection of messages of abnormal lengths suggested that these mutations did not lead to complete loss of transcripts due to non–sense-mediated decay.

Analysis of cDNA for Loss of Expression

For each gene, all probands whose genomic DNA was heterozygous for at least 1 exonic polymorphism were assessed for heterozygosity at the cDNA level. The cDNA was amplified with PCR primers flanking the polymorphism and spanning at least 1 intron, then sequenced to determine if both alleles were expressed in the transcript. Heterozygous individuals with only 1 allele revealed in their cDNA were candidates for rearrangements involving upstream regulatory domains. For all such events in *BRCA1* and *BRCA2*, multiplex ligation-dependent probe amplification (MLPA) also detected a deletion beginning upstream of exon 1 of either *BRCA1* or *BRCA2*.

Analysis of Potential Exonic Splice Enhancers

Complete DNA sequencing of *BRCA1*, *BRCA2*, and *CHEK2* identified multiple rare variants of unknown significance in the coding sequences of both genes. All such variants were tested for their potential to alter splicing.[43-46] Assessment was first carried out based on exonic splice enhancer motif scores calculated by ESEfinder 2.0[47] then by experimental tests of splicing of all rare variants with suggestive motif scores. PCR was performed with primers flanking the exon with the genomic variant and spanning at least 1 intron. The PCR products from patient cDNA were sequenced to determine if both alleles were wild type and expressed at similar levels. Length was also assessed by agarose electrophoresis. For *BRCA2*.459T→G, located in exon 3, cDNA analysis was complicated by the naturally occurring mRNA isoform lacking exon 3. In this case we analyzed *BRCA2*.459T→G by hybrid minigene, as has been described.[48]

Analysis of Copy Number Using MLPA

BRCA1, *BRCA2*, and *CHEK2* copy number was quantified by MLPA (MRC-Holland, Amsterdam, the Netherlands) using kit P087, which includes probes for each of the 24 exons of *BRCA1*,[49] and kit P045, which includes probes for 23 of the 27 exons of *BRCA2* and for exon 9 of *CHEK2*. MLPA was performed in duplicate for each sample on an ABI 2700 thermal cycler (Applied Biosystems, Foster City, Calif). Each MLPA analysis was carried out on 10 test samples and 2 positive controls (a deletion and a duplication), then electrophoresed on an ABI 3100 genetic analyzer (Applied Biosystems) and interpreted using Genescan version 3.1 (Applied Biosystems). Peak heights were exported to an Excel spreadsheet, and ratios of each test peak relative to all the other peaks for that individual were assessed. Probes with a dosage quotient less than 0.7 (for deletions) or greater than 1.2 (for duplications) were tested again. In our experience, false-positive deletions or duplications of single exons were common but resolved by repeated testing. Rearrangements of contiguous exons rarely arose as false-positive results. In 2 cases, a false-positive result was obtained because of a benign polymorphism, *BRCA2*.2192C→G (P655R), at the exon 11 probe binding site.

Identification of Genomic Breakpoints and Development of Diagnostic Primers

Putative deletions and duplications detected by cDNA analysis, MLPA, or both were evaluated in genomic DNA by long-range PCR (Expand System; Roche Applied Science, Indianapolis, Ind). The PCR primers were located in exons flanking the rearrangement, and PCR products of abnormal size were sequenced in both directions. Sequence traces were aligned to the May 2004 assembly of the Human Genome Browser (available at http://genome/ucsc.edu). The RepeatMasker program (available at http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker) was used to identify *Alu* sequences at breakpoint junctions. To characterize deletions in promoters and 5′ UTRs, we genotyped single nucleotide polymorphisms (SNPs) and microsatellite markers to identify possible inherited loss of heterozygosity. The PCR primers were then designed to flank hemizygous regions and long-range PCR performed to determine the sizes of the genomic regions. If genomic products of only expected sizes were obtained, these were sequenced until evidence of heterozygosity was documented, demonstrating that results of cDNA analysis represented naturally occurring splice isoforms or that MLPA results represented false-positive results. If genomic products of abnormal sizes were obtained, these were sequenced to identify the breakpoints.

Screening for the *CHEK2* Deletion in Czech and Slovak Patients

Genomic DNA from 3 hospital-based series of patients with breast cancer from the Czech and Slovak Republics was amplified with primers flanking the deletion identified in the families of Czechoslovakian ancestry: 5′-GAACCACTATTTACATAAC-3′ and 5′-GTCTCAAACTTGGCTGCG-3′. These

Table 1. Characteristics of Families With ≥4 Cases of Breast or Ovarian Cancer With Negative (Wild-type) Genetic Test Results for *BRCA1* and *BRCA2* (N = 300)

Characteristic	No. (%)
Cancer type	
Female breast, age at index diagnosis, y	291 (97)
<30	14 (5)
30-39	77 (26)
40-49	127 (42)
50-59	67 (22)
≥60	6 (2)
Ovarian	6 (2)
Male breast	3 (1)
Breast or ovarian cancers in the family	
Female breast only	140 (47)
Female breast and ovary	135 (45)
Female breast and male breast	13 (4)
Female breast and ovary and male breast	12 (4)
Syndromic features	
Li-Fraumeni	3 (1)
Li-Fraumeni–like	7 (2)
Cowden	8 (3)
None apparent	282 (94)
Ancestry*	
European	285 (95)
Latin American	6 (2)
African American	4 (1)
American Indian, Inuit	3 (1)
East Asian	2 (1)

*Self-defined by participants.

primers yielded a product of 1.8 kb for the mutant *CHEK2* allele. Primers 5′-AGAACCACTATTTACATAAC-3′ and 5′-CAAGACACTGAAAGATACTC-3′ amplify a product of 1.2 kb for the wild-type *CHEK2* allele and were used to test DNA integrity. From all individuals with apparently positive test results for the deletion, DNA was sequenced to confirm the breakpoints.

Analysis of *CHEK2* Haplotypes and Loss of Heterozygosity in Tumors

Samples carrying the *CHEK2* genomic deletion were genotyped for microsatellites D22S689, D22S275, and D22S1150 and for a previously uncharacterized TG_{20} repeat located in intron 6 of *CHEK2*. Genotyping was carried out using radiolabeled PCR and polyacrylamide gel electrophoresis.[13] Loss of heterozygosity was assessed in DNA from tumor samples at informative microsatellites. Radiolabeled PCR products from tumor and germline DNA were evaluated for loss of heterozygosity as previously described.[13]

RESULTS

Characteristics of the 300 probands in the study and their families are indicated in TABLE 1. Of the probands, 291 were females with invasive breast cancer, 3 were males with invasive breast cancer, and 6 were females with ovarian cancer. All represented families with at least 4 cases of breast cancer, ovarian cancer, or both. Ages at breast cancer diagnosis of probands were younger than average ages at diagnosis for their families, because within a family at risk, patients with earlier-onset breast cancer are most likely to be tested. Ancestries were based on self-report. Ashkenazi Jewish ancestry was reported by 31 probands.

Families included at least 4 cases of breast or ovarian cancer but were not selected specifically for the occurrence of ovarian cancer or male breast cancer. Nonetheless, 53% of families included one or both of these cancers, probably because we enrolled many extended kindreds. The immediate family of a proband generally included few affected relatives and no cases of ovarian cancer. It is important to take family size into consideration when applying the results of this study to small families encountered in clinical practice. That is, the absence of ovarian cancer in a small family may simply reflect few older women in a family, rather than the absence of any predisposition to ovarian cancer.

All probands were screened for inherited rearrangements of *BRCA1*, *BRCA2*, and *CHEK2* by MLPA using genomic DNA extracted from fresh blood. In addition, full-length cDNA copies of *BRCA1*, *BRCA2*, and *CHEK2* were generated from RNA of all probands and screened for length variation, for loss of genomic heterozygosity in transcripts, and for splicing alterations introduced by potential exon splice enhancer variants. *CHEK2* was fully sequenced from genomic DNA of all probands, and *CHEK2* exon 9 was screened by MLPA. *TP53* was fully sequenced from genomic DNA of 31 probands, 10 from families with Li-Fraumeni features[50] and 21 from other families with at least 2 cases of breast cancer diagnosed prior to age 35 years. The coding exons, 5′ UTR and 3′ UTR of *PTEN*, were fully sequenced from genomic DNA of 8 probands of families with features of Cowden syndrome.[51]

Of the 300 families, 52 (17%) carried inherited mutations in *BRCA1*, *BRCA2*, *CHEK2*, or *TP53*. Mutation-positive families included 31 with rearrangements of *BRCA1*, 4 with rearrangements of *BRCA2*, 14 with mutations in *CHEK2*, and 3 with mutations in *TP53* (TABLE 2). No inherited mutations were detected in *PTEN*. Inherited rearrangements of *BRCA1* were more frequent among probands diagnosed when younger than 40 years (15/91 [16%]) than among probands diagnosed when 40 years or older (13/200 [6.5%]) ($P = .007$). Inherited rearrangements of *BRCA1* and *BRCA2* were found in a larger proportion of families including cases of ovarian cancer, male breast cancer, or both (29/160 [18%]) than of families with only female breast cancer (6/140 [4.2%]) ($P < .001$). These results are consistent with associations observed for conventional mutations in *BRCA1* and *BRCA2*. Genomic deletions of *BRCA2* have been previously reported in families with male breast cancer.[32] Our results extend this observation to families with no cases of male breast cancer. We identified 2 deletions and a duplication of regions of *BRCA2* in 4 families, 3 of which included no cases of male breast cancer. Among the probands of Ashkenazi Jewish ancestry, no *BRCA1* or *BRCA2* genomic rearrangements were identified, and 4 patients carried mutations in *CHEK2*.

The spectrum of *BRCA1* and *BRCA2* mutations in these families is very broad (TABLE 3). The 52 mutation-positive families harbored 28 different mutations. All genomic deletions and duplications were individually rare, reflected in the observation that 14 of the 22 rearrangements are not among the large number of these mutations previously reported.[36] In our view, all potential deletions and duplications should be confirmed with diagnostic

primers in patients' genomic DNA, because MLPA used alone can yield false-positive assessments of copy number. The set of diagnostic primers used is available from the authors on request.

At *BRCA1* and *BRCA2*, genomic deletions and duplications varied in size from less than 1 kb to greater than 100 kb and appeared at all regions of *BRCA1* and *BRCA2* (FIGURE). Deletions of promoter regions led to no detectable expression of message. Intragenic rearrangements led either to premature protein truncations or to in-frame duplications or deletions. Families with mutations of different effects—eg, loss of transcript expression, premature stops, or in-frame deletions or duplications—did not differ in age at breast cancer diagnosis or in family history of ovarian cancer or male breast cancer. Breakpoints of most rearrangements occurred in *Alu* repeats, perhaps reflecting errors in homologous recombination as the mechanism of mutagenesis.[52,53]

Both the DNA-based MLPA and the cDNA-based analyses were effective in detecting genomic rearrangements. Screening cDNA for loss of transcript expression was not informative for 6 probands who were homozygous for all exonic SNPs, but MLPA was informative in detecting promoter deletions in these probands. In addition to being informative for nearly all types of mutations, MLPA has the advantage that the analysis is carried out on genomic DNA, eliminating the need for cell lines or RNA isolation.

At *CHEK2*, complete sequence was determined for all 300 probands, and mutations of all types were scored. Three point mutations of *CHEK2* were observed: 1100delC in 3 families, S428F in 2 families, and I157T in 1 family. Mutations in *CHEK2* were not significantly associated with age at breast cancer diagnosis of the index case or with family history of male breast cancer or ovarian cancer (Table 2). *CHEK2*.1100delC and *CHEK2*.S428F each increase risk of female breast cancer approximately 2-fold.[11-13] Some studies have found no association between *CHEK2*.I157T and risk of female breast cancer; other studies estimate the odds ratio for *CHEK2*.I157T to be between 1.4 and 3.6.[54-60] In the one family in our series in which it appeared, *CHEK2*.I157T was inherited by 5 of 6 patients with breast cancer, while all unaffected women older than 40 years had wild-type *CHEK2* sequences.

We also discovered a *CHEK2* genomic deletion that is a founder mutation among patients of Czechoslovakian ancestry with breast cancer. Two probands carried a *CHEK2* genomic deletion of 5567 bp, leading to loss of exons 9 and 10 and predicted protein truncation at codon 381 (Table 3). In each of these families, the deletion appeared both in women with breast cancer and in those with ovarian cancer. Because these families were of Czechoslovakian ancestry, we tested whether this deletion was associated with breast cancer in the Czech and Slovak populations generally. We genotyped *CHEK2*.del5567 in 3 independent series of patients with breast cancer and female controls from the Czech and Slovak Republics. The deletion was present in 8 of 631 patients (1.3%) with invasive breast cancer (5/201 [2.5%] in Prague, 2/349 [0.57%] in Brno, and 1/81 [1.2%] in Bratislava) but in 0 of 367 controls ($P = .03$ by Fisher exact test). In all patients, *CHEK2*.del5567 appeared on the same 650-kb haplotype defined by microsatellite markers D22S689, D22S275, and D22S1150, indicating that the mutation had a single source. Family ancestries suggest that the

Table 2. Frequencies of Inherited Genomic Rearrangements in *BRCA1* and *BRCA2* and of All Inherited Mutations in *CHEK2* and *TP53*

Characteristic of Family	No. of Patients Tested	Gene With Inherited Mutation				
		BRCA1	*BRCA2*	*CHEK2*	*TP53*	Total, No. (%)
Proband cancer type						
Female breast, age at diagnosis, y	291	28	3	13	3	47 (16)
<30	14	3	0	0	3	6 (43)
30-39	77	12	1	6	0	19 (25)
40-49	127	12	1	4	0	17 (13)
50-59	67	1	1	3	0	5 (7)
≥60	6	0	0	0	0	0
Ovarian	6	2	1	0	0	3 (50)
Male breast	3	1	0	1	0	2 (67)
Breast or ovarian cancers in the family						
Female breast only	140	5	1	9	2	17 (12)
Female breast and ovary	135	23	2	3	1	29 (21)
Female breast and male breast	13	2	0	1	0	3 (23)
Female breast and ovary and male breast	12	1	1	1	0	3 (25)
Syndromic features						
Li-Fraumeni	3	0	0	0	2	2 (67)
Li-Fraumeni–like	7	0	0	0	1	1 (14)
Cowden	8	0	0	0	0	0
Total	300	31	4	14	3	52 (17)

mutation originated in the Carpathian Mountains on the border of the present Czech and Slovak Republics. In addition, breast tumors from 2 patients revealed loss of the wild-type allele of *CHEK2*, so that only the allele with the deletion was present in cancer cells. Of the 8 Czech and Slovak patients with the deletion, 4 reported breast cancer in their mothers, but none reported ovarian cancer in mothers, sisters, or aunts.

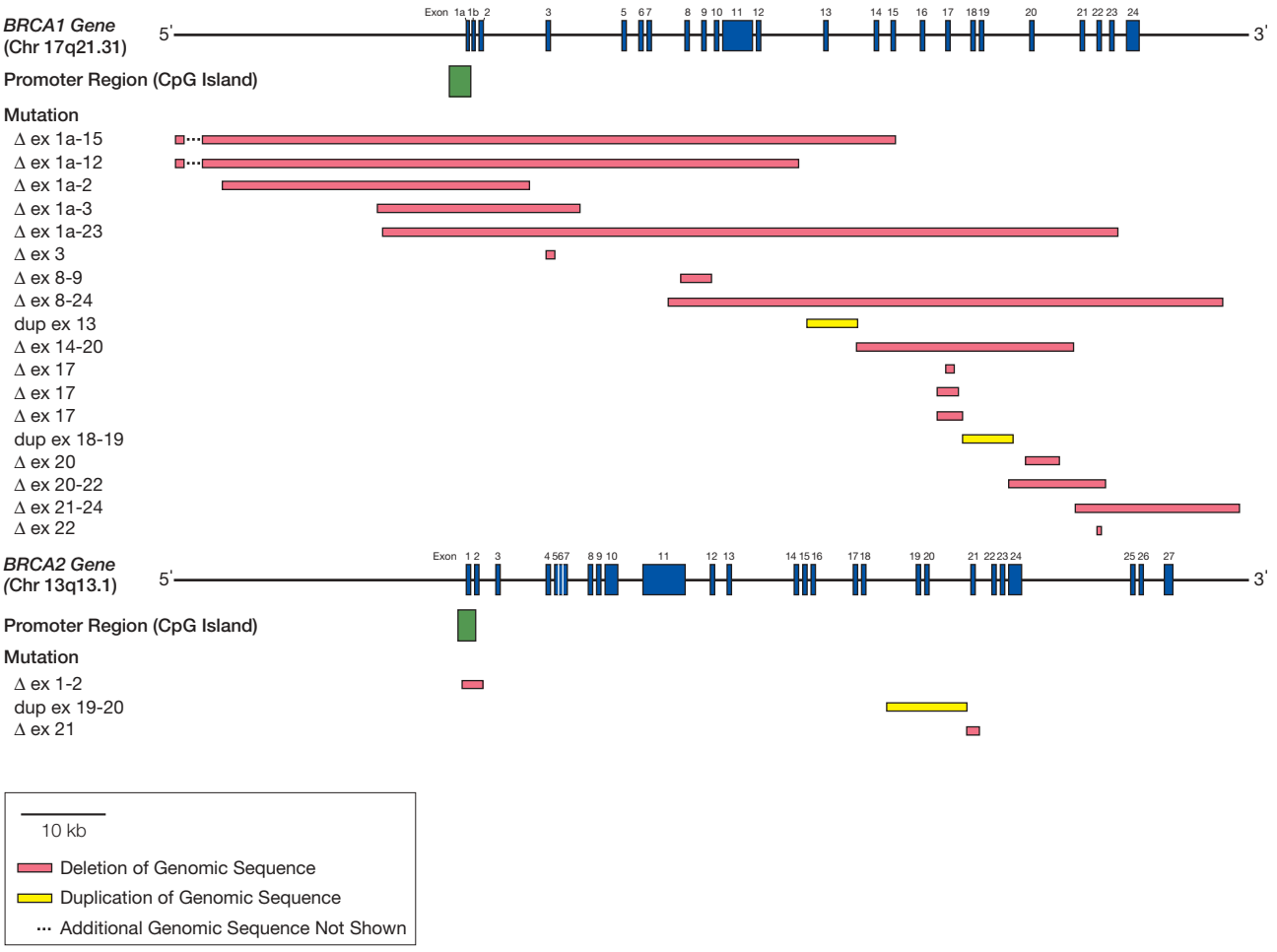
Inherited mutations in *TP53* appeared in 2 of 3 families with Li-Fraumeni syndrome and in 1 of 7 families with Li-Fraumeni–like syndrome (TABLE 4). We also sequenced *TP53* in genomic DNA from probands of 21 other families with 2 or more cases of breast cancer younger than 35 years but no cases of soft tissue sarcoma, osteosarcoma, brain tumor, leukemia, or adrenocortical carcinoma. *TP53* was wild type in these 21 families. We did not detect mutations in *PTEN* in any of the 8 families with signs of Cowden syndrome, although we previously detected inherited *PTEN* mutations in other families in our series.[16] The families with Cowden syndrome in the pres-

Table 3. Inherited Genomic Rearrangements in *BRCA1* and *BRCA2* and All Inherited Mutations in *CHEK2* and *TP53* in 300 US Families at High Risk of Breast Cancer and With Negative (Wild-type) Results by Conventional Genetic Testing

Mutation	Size, bp	Method of Detection			Genomic Breakpoints*		Effect of Mutation	Type of Sequence at Breakpoint		Ancestries of Families With Mutation
		GS	MLPA	cDNA	Centromeric	Telomeric		5′ Site	3′ Site	
						BRCA1				
Δ ex 1a-15†	del >169639‡	No	Yes	Yes	17:38476471 –17:38480064	17:38749703 –17:38773360	No transcript			German
Δ ex 1a-12†	del 88550	No	Yes	Yes	17:38495935	17:38584485	No transcript	*Alu*Sx	*Alu*Jo	Irish
Δ ex 1a-2	del 36934	No	Yes	No	17:38525763	17:38562118	No transcript	Duplicated region	Duplicated region	German
Δ ex 1a-3†	del 23395	No	Yes	No	17:38519865	17:38543865	No transcript	*Alu*Sx	*Alu*J	Norwegian
Δ ex 1a-23†	del 86853	No	Yes	Yes	17:38451657	17:38538510	No transcript	*Alu*Sx	*Alu*Y	Irish
Δ ex 3	del1049 ins7	No	Yes	Yes	17:38520262	17:38521304	Stop codon 27	Single copy	Single copy	English
Δ ex 8-9†	del 3936	No	Yes	Yes	17:38502281	17:38506245	Stop codon 164	(TA)n	Single copy	African American
Δ ex 8-24†	del 65520	No	Yes	Yes	17:38441660	17:38507180	Stop codon 148	*Alu*Sx	*Alu*Jo	German
dup ex 13	dup 6081	No	Yes	Yes	17:38483825	17:38489905	Stop codon 1460	*Alu*Sx	*Alu*Sx	Northern European
Δ ex 14-20	del 26456	No	Yes	Yes	17:38457242	17:38483691	Deletion of 307 aa	*Alu*Sx	*Alu*Sg	Irish
Δ ex 17 (1.0 kb)	del 1016	No	Yes	Yes	17:38472804	17:38473817	Stop codon 1672	*Alu*Y	*Alu*Sc	German
Δ ex 17 (2.7 kb)†	del 2680	No	Yes	Yes	17:38473067	17:38475745	Stop codon 1672	*Alu*Sp	*Alu*Sc	German
Δ ex 17 (3.1 kb)	del 3118	No	Yes	Yes	17:38472400	17:38475516	Stop codon 1672	*Alu*Sp	*Alu*Sq	Irish, Swedish, Norwegian
dup ex 18-19†	dup 5923	No	Yes	Yes	17:38466304	17:38472226	Duplication of 40 aa	*Alu*Sg	*Alu*Sx	African American
Δ ex 20†	del 3985	No	Yes	Yes	17:38458944	17:38462928	Deletion of 28 aa	*Alu*Sx	*Alu*Y	French
Δ ex 20-22†	del 11357	No	Yes	Yes	17:38453698	17:38465050	Deletion of 71 aa	(CCA)n	*Alu*Jo	English
Δ ex 21-24†	del 19245	No	Yes	Yes	17:38438101	17:38457346	Stop codon 1778	*Alu*Sg	*Alu*Sp	Irish
Δ ex 22	del 510	No	Yes	Yes	17:38454264	17:38454773	Stop codon 1804	Single copy	*Alu*Sx	Dutch
						BRCA2				
Δ ex 1-2	del 2352 ins 12	No	Yes	Yes	13:31786709	13:31789062	No transcript	*Alu*Sp	*Alu*Sx	English
dup ex 19-20†	dup 9700	No	Yes	Yes	13:31838735	13:31848434	Stop codon 2957	*Alu*Sx	*Alu*Sx	Dutch, German
Δ ex 21†	del 1518	No	Yes	Yes	13:31848537	13:31850057	Stop codon 2958	*Alu*Sx	Single copy	Hungarian
						CHEK2				
Δ ex 9-10†	del 5567	No	Yes	Yes	22:27416941	22:27422508	Stop codon 381	*Alu*Jo	*Alu*Sq	Czech, Slovak
470 C→T	Missense	Yes	No	No			Ile 157 Thr			Scandanavian
1100 ΔC	del C	Yes	No	No			Stop codon 381			Northern European
1283 C→T	Missense	Yes	No	No			Ser 428 Phe			Ashkenazi Jewish
						TP53				
733 G→A	Missense	Yes	No	No			Gly 245 Ser			Brazilian
524 G→A	Missense	Yes	No	No			Arg 175 His			Mexican
831 T→A†	Nonsense	Yes	No	No			Stop codon 277			English

Abbreviations: aa, amino acid(s); cDNA, complementary DNA; Δ, deletion of exon(s); del, deletion of genomic sequence; dup, duplication of genomic sequence; ex, exon(s); GS, genomic sequencing; ins, insertion of genomic sequence; MLPA, multiplex ligation-dependent probe amplification.
*Genomic locale from the Human Genome Browser, May 2004 assembly (available at http://genome.ucsc.edu/).
†Mutation not previously reported.
‡Exact size not determinable because this deletion spans a still-uncharacterized gap in the consensus human genome sequence; genomic breakpoints are between the sites indicated.

Figure. Inherited Genomic Rearrangements of *BRCA1* and *BRCA2*



Chr indicates chromosome; Δ, deletion of exon(s); dup, duplication of genomic sequence; ex, exon(s).

ent cohort may harbor mutations in the *PTEN* promoter region, which we did not sequence.[61]

COMMENT

The US Preventive Services Task Force recently recommended that genetic testing be offered to women whose family histories suggest inherited *BRCA1* or *BRCA2* mutations.[62] Identification of inherited *BRCA1* and *BRCA2* mutations enables carriers to undertake individualized cancer prevention strategies, including consideration of breast magnetic resonance imaging and of risk-reducing salpingo-oophorectomy and prophylactic mastectomy.[21-27] Clearly, the surgical interventions are highly

Table 4. Families With Li-Fraumeni Syndrome (LFS) or Li-Fraumeni–Like Syndrome (LFL) and ≥4 Cases of Breast Cancer and/or Ovarian Cancer

	Soft Tissue Sarcoma, Osteosarcoma, Brain Tumor, Leukemia				
Family	Type	Ages at Diagnosis, y	Familial Young Breast Cancer Cases by Ages at Diagnosis, y	Syndrome	*TP53* Sequence
86	Rhabdomyosarcoma	4	21, 27	LFS	733 G→A(Gly245Ser)
	Leukemia	14, 18			
168	Brain	5	25,25,26, 26	LFL	831 T→A(Cys277stop)
450	Osteosarcoma	26	32,42,42, 44	LFS	Wild type
552	Brain	11	39, 44	LFL	Wild type
710	Brain	39	32, 36	LFL	Wild type
787	Sarcoma	29	25,29,31,34,36	LFL	Wild type
794	Brain	9	37	LFL	Wild type
796	Leukemia	4	40, 44	LFL	Wild type
825	Brain	30	40,41,43, 43	LFL	Wild type
861	Sarcoma	3	26, 35	LFS	524 G→A(Arg175His)

invasive; risk-reducing salpingo-oophorectomy, in particular, effectively decreases the risk of both ovarian and breast cancer in mutation carriers but is accompanied by abrupt surgical menopause.[25,26] Increasing evidence for the efficacy of these risk-reduction strategies in high-risk women has increased demand for genetic testing. Women at high risk and their clinicians want accurate assessment of genetic risk prior to embarking on such invasive and expensive risk management options.

Our results suggest that genetic testing, as currently carried out in the United States, does not provide all available information to women at risk. Our data indicate that 12% of those from high-risk families with breast cancer and with negative (wild-type) commercial genetic test results for *BRCA1* and *BRCA2* nonetheless carry cancer-predisposing genomic deletions or duplications in one of these genes. Our results are consistent with previous studies specifically of *BRCA1* in various European populations and in a smaller series of US families using earlier tools.[29-36,49,63]

The clinical dilemma is what to offer to women with a high probability of carrying a mutation in *BRCA1* or *BRCA2* but with negative commercial test results. Technically, the answer is at hand. The mutations identified in our study that were missed by commercial testing are detectable using other approaches that are currently available. There are thousands of different mutations leading to breast cancer predisposition, including many *BRCA1* and *BRCA2* rearrangements, which differ in size and locale and are individually rare. Therefore, a generic method should be used to detect them. Mutation-specific primers are useful but will not detect all rearrangements. All genomic alterations in our series were identified by MLPA, which allows rapid and cost-effective analysis of rearrangements across the entire *BRCA1* and *BRCA2* genes. We believe that for families testing negative (wild type) for *BRCA1* and *BRCA2* by conventional sequencing, MLPA followed by sequence confirmation of breakpoints in patients' genomic DNA is the current best choice for evaluating the wide range of genomic rearrangements in *BRCA1* and *BRCA2*. It is important to note that MLPA cannot substitute for sequencing, because the 2 methods detect different classes of mutations. Both sequencing and global screening for rearrangements are necessary, and MLPA should be carried out for those families with wild-type test results based on sequencing alone.

Clinical testing options for *BRCA1* and *BRCA2* are limited in the United States. In contrast to genetic testing for *BRCA1* and *BRCA2*, genetic testing for other cancer susceptibility genes (*MSH2*, *MLH1*, *PTEN*, *TP53*, etc) is available from numerous profit and not-for-profit laboratories, with a range of testing options and prices. Although MLPA appears to be the most efficient and cost-effective strategy to identify genomic rearrangements in *BRCA1* and *BRCA2*, clinical testing using MLPA is not available in the United States. For those women who test negative for rearrangements in *BRCA1* and *BRCA2*, participation in research studies is not an adequate substitute for providing the most effective and thorough clinical genetic testing.

In addition to DNA sequencing of *BRCA1* and *BRCA2*, genetic testing for other major breast cancer susceptibility genes including *CHEK2*, *PTEN*, and *TP53* is clinically available in the United States. Our data help clarify when testing for those genes is likely to yield positive results. In our series of patients with wild-type *BRCA1* and *BRCA2* test results, 4% of patients carried inherited mutations in *CHEK2* and 1% of patients carried inherited mutations in *TP53*. The clinical implications of inherited mutations in these 2 genes are very different. Persons with inherited *TP53* mutations have a greater than 90% risk of developing one of the cancers associated with the Li-Fraumeni syndrome.[64] Fortunately, inherited mutations in *TP53* are rare, in our series appearing only in families that met criteria for Li-Fraumeni syndrome or Li-Fraumeni–like syndrome. No *TP53* mutations were identified in 21 families with very early onset breast cancer (<35 years) but without other Li-Fraumeni tumors.

In contrast, inherited mutations in *CHEK2* are less severe, leading to an approximately 2-fold increase in risk of breast cancer.[11-13] Previous evidence suggested that mutations in *CHEK2* also increase risk for prostate and thyroid cancers.[55,56,65-67] Our analyses of the *CHEK2* deletion in the Czech and Slovak populations are consistent with a 2-fold increased risk of breast cancer. Our results also suggest that the full mutational spectrum of *CHEK2* has not yet been revealed. In particular, the MLPA kit does not yet include probes to test for duplications or deletions of all exons of *CHEK2*.

The usefulness of clinical testing for *CHEK2* mutations has been debated. It is uncertain whether mutations in *CHEK2* alone can account for breast cancer clustering in the families in our series. In severely affected families, it is possible that other genetic factors interact with a *CHEK2* mutation to increase risk.[68] In a family with 4 or more breast cancers and a known *CHEK2* mutation, one cannot be certain that a woman with wild-type *CHEK2* has no more than a population-level risk of breast cancer. Conversely, in a severely affected family, a woman who carries the familial *CHEK2* mutation might have more than a 2-fold increased risk of breast cancer. Prospective studies of families with *CHEK2* mutations are needed to determine cancer risks and allow more accurate counseling.

In families with few cases of breast cancer, the risk associated with *CHEK2* mutations is likely to reflect the 2-fold increase observed in the general population. In such families, negative test results for *CHEK2* are reassuring. Another role for *CHEK2* testing lies in understanding cancer risk to other organs. A breast cancer survivor with a *BRCA1* or *BRCA2* mutation has a high risk of ovarian cancer. In contrast, a woman with a *CHEK2* mutation and no family history of ovarian cancer would

not be presumed to have an elevated risk of ovarian cancer. *CHEK2* testing may also prove useful to clinical trials for cancer risk screening or prevention. Understanding the efficacy of interventions such as breast magnetic resonance imaging in *CHEK2* mutation carriers will help define the role of various cancer risk strategies in these women. As risk reduction options become more extensive and varied, risk stratification is increasingly important.

This study's families were selected to include 4 or more cases of breast or ovarian cancer and hence are very likely to have inherited genetic predisposition for breast cancer. Despite this selection criterion and the extensive testing used, the high risk posed to many families remains unexplained. This is particularly true of families with female breast cancer at older ages of diagnosis and without ovarian cancer or male breast cancer. We are actively searching for other genes responsible for cancer in these families. As more breast cancer susceptibility genes of different penetrances are identified, clinicians will be increasingly challenged to offer the most appropriate genetic tests, to assist patients in interpreting the results, and to optimize risk reduction strategies.

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