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Germline *BRCA1* alterations in a population-based series of ovarian cancer cases

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The objective of this study was to provide more accurate frequency estimates of breast cancer susceptibility gene 1 (*BRCA1*) germline alterations in the ovarian cancer population. To achieve this, we determined the prevalence of *BRCA1* alterations in a population-based series of consecutive ovarian cancer cases. This is the first population-based ovarian cancer study reporting *BRCA1* alterations derived from a comprehensive screen of the entire coding region. One hundred and seven ovarian cancer cases were analyzed for *BRCA1* alterations using the RNase mismatch cleavage assay followed by direct sequencing. Two truncating mutations, 962del4 and 3600del11, were identified. Both patients had a family history of breast or ovarian cancer. Several novel as well as previously reported uncharacterized variants were also identified, some of which were associated with a family history of cancer. The frequency distribution of common polymorphisms was determined in the 91 Caucasian cancer cases in this series and 24 sister controls using allele-specific amplification. The rare form of the Q356R polymorphism was significantly ($P = 0.03$) associated with a family history of ovarian cancer, suggesting that this polymorphism may influence ovarian cancer risk. In summary, our data suggest a role for some uncharacterized variants and rare forms of polymorphisms in determining ovarian cancer risk, and highlight the necessity to screen for missense alterations as well as truncating mutations in this population.

INTRODUCTION

Ovarian cancer accounts for 4% of all cancers in women. In 1997, ~26 700 new cases were diagnosed and ~14 800 women died of the disease (1). The most significant risk factor for ovarian cancer development is a family history of breast and/or ovarian cancer. An estimated 5–10% of all ovarian cancer cases are hereditary (2).

Early genetic linkage studies suggested that frameshift mutations that lead to premature truncation of the protein, as well as missense mutations that generate Stop codons leading to premature protein truncation, account for 45% of hereditary breast cancer and 80–90% of breast/ovarian cancer cases (3,4). Based on these studies, it was determined that breast cancer susceptibility 1 (*BRCA1*) mutation carriers had a lifetime risk of 85% for breast cancer and 63% for ovarian cancer. More recently, larger studies of women that were not highly selected for increased risk suggest that risk estimates are far lower and that mutations in *BRCA1* contribute to a lower proportion of women with a family history of breast or ovarian cancer (5,6).

Over 500 alterations in the *BRCA1* gene have been documented (7). Most of these are frameshifts and ~10% are missense mutations that generate Stop codons. There is, however, a significant number of single amino acid changes that have been identified and classified as either uncharacterized variants or common polymorphisms according to the frequency of the less common allele and its occurrence in any given population. The significance of these uncharacterized variants and common polymorphisms with respect to ovarian cancer risk is, at present, not known. It is of note that comparative studies with *Brca1*, the mouse homolog of *BRCA1*, have revealed that, of the missense changes studied, the majority of uncharacterized variants occurred within amino acids that were conserved between the two species (8,9), suggesting functional conservation. In contrast, most of the amino acids involved in polymorphic variations were not conserved (8). In addition, our group has provided evidence that the R841W missense alteration is associated with a family history of breast and ovarian cancer displaying a later age of onset (10). This suggests that a certain proportion of uncharacterized variants may affect BRCA1 function and increase breast and ovarian cancer risk.

Frequency estimates of *BRCA1* alterations in ovarian cancer populations have been derived mainly from studies of high risk

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cancer families. However, because these families were selected based on multiple breast and ovarian cancer cases, these estimates may not be representative of the general population. The objective of this study was to provide more accurate frequency estimates of these alterations in the ovarian cancer population. We report the frequency of *BRCA1* frameshift mutations in a population-based series of 107 ovarian cancer cases and provide evidence that some uncharacterized variants and rare forms of polymorphism may influence ovarian cancer risk.

RESULTS

Subjects

The study included 107 consecutive population-based cases of ovarian cancer diagnosed between March 1, 1994 and February 28, 1995 in Orange County, CA. The participation rate was 82%. All women who developed ovarian cancer during the recruitment period were eligible for inclusion in the study. The study included all subjects for which sufficient DNA and RNA were available for analysis. There were no exclusion criteria. The age of cancer onset among participants and non-participants was as follows: for age intervals of <40, 40–49, 50–59, 60–69 and >70 years, the age distribution of participants was 16, 20, 20, 22 and 22%, respectively. The age distribution for non-participants was 14, 10, 11, 29 and 36%, respectively. There were no significant differences in age distribution between the participants and non-participants. The mean age at cancer onset among probands was 55.04 ± 15.4 years, and 38% were younger than 50 years of age. The majority of cases (85%) were non-Hispanic white, 9% were Hispanic and 6% were Asian. The mean age at onset among non-Hispanic whites (57.1 ± 14.5) was significantly higher than that of non-whites (43.4 ± 15.7; $P < 0.0008$). To reduce bias due to race, the description of clinical and demographic characteristics of cases and the analyses of common polymorphisms were limited to Caucasians only (91 cases and 24 controls). Thirty older unaffected sisters were also recruited into the study.

Table 1. Characteristics of the ovarian cancer cases and of first and second degree affected relatives with respect to age at onset

	n (%)	Age at onset		
		Range	Mean ± SD	95% CI[a]
Probands				
Family history of breast or ovarian cancer				
Positive	16 (18)	39–67	51.3 ± 8.3	(46.9, 55.7)
Negative	73 (82)	21–84	58.0 ± 15.2	(54.5, 61.5)
Family history of breast cancer only				
Positive	12 (13)	39–67	50.8 ± 8.8	(45.2, 56.4)
Negative	77 (87)	21–84	57.7 ± 14.9	(54.4, 61.0)
Family history of ovarian cancer only				
Positive	6 (7)	39–60	49.5 ± 8.1	(41.0, 58.0)
Negative	83 (93)	21–84	57.3 ± 14.6	(54.2, 60.4)
Relatives with ovarian cancer				
Family history of breast or ovarian cancer				
Positive (first and second degree)	5 (71)	50–67	57.2 ± 6.2	(49.5, 64.9)
Negative (second degree only)	2 (29)	45–48	46.8 ± 2.5	(24.3, 69.3)
Family history of breast cancer only				
Positive (first and second degree)	1 (14)	67.0		
Negative (second degree only)	6 (86)	45–57	52.1 ± 5.0	(46.9, 57.3)
Family history of ovarian cancer only				
Positive (first and second degree)	5 (71)	50–67	57.2 ± 6.2	(49.5, 64.9)
Negative (second degree only)	2 (29)	45–48	46.8 ± 2.5	(24.3, 69.3)
Relatives with breast cancer				
Family history of breast or ovarian cancer				
Positive (first and second degree)	13 (41)	37–81	57.0 ± 13.3	(49.0, 65.0)
Negative (second degree only)	19 (59)	26–80	55.1 ± 13.6	(48.5, 61.7)
Family history of breast cancer only				
Positive (first and second degree)	12 (38)	37–81	57.7 ± 13.6	(49.1, 66.3)
Negative (second degree only)	20 (62)	26–80	57.7 ± 13.6	(48.5, 60.9)
Family history of ovarian cancer only				
Positive (first and second degree)	3 (9)	37–66	50.3 ± 14.6	(14.0, 86.6)
Negative (second degree only)	29 (91)	26–81	56.4 ± 13.3	(51.3, 61.5)

[a]95% confidence interval.

Approximately 13% of cases reported a family history of only breast cancer in at least one first degree relative, while 7% reported a family history of only ovarian cancer among first degree relatives. Two cases were excluded from the classification of family history because the probands were adopted. There were no significant associations with age of diagnosis and a positive or negative family history of breast or ovarian cancer among the 89 Caucasian probands, or among their affected first and second degree relatives (Table 1). No significant trends were observed with respect to age at diagnosis when cases were classified by laterality, differentiation, histology or presence of multiple primaries. The distribution of first and second degree relatives with breast or ovarian cancer in the probands' families is presented in Table 2. Three ovarian cancer probands were of Ashkenazi Jewish descent. Of these, two probands had no family history and one had a mother who developed ovarian cancer at 50 years of age.

Table 2. Distribution of first and second degree relatives with breast or ovarian cancer in each proband's family

Number of probands (%)	Number of first degree relatives	Number of second degree relatives
57 (64)	0	0
14 (16)	0	1
2 (2)	0	2
13 (15)	1	0
1 (1)	1	2
2 (2)	2	0

BRCA1 mutations

All 107 ovarian cancer cases were analyzed for germline *BRCA1* mutations using the RNase mismatch cleavage assay (NIRCA). Two *BRCA1* frameshift mutations were identified, 962delCTCA (codon 281) and 3600delGAAGATACTAG (codon 1161). Both of these occurred in Caucasians with a positive family history of breast or ovarian cancer in a first degree relative. The proband with the 3600del11 mutation had a mother with ovarian cancer at age 67 years, a sister with breast cancer at age 48 years, a maternal grandmother with breast cancer at age 78 years and a maternal aunt with breast cancer at age 72 years. The proband with the 962del4 mutation had a mother with ovarian cancer at age 49 years, a paternal half-sister with breast cancer at age 48 years and ovarian cancer at age 55 years and a maternal aunt with breast cancer at age 52 years. Other cancers in first and/or second degree relatives included malignant melanoma, pancreatic, stomach and prostate cancers. The two *BRCA1* mutation carriers were not significantly younger than non-carriers (mean age at onset 48.0 years for the *BRCA1* mutation carriers compared with 57.3 years for the non-carriers). Tumor pathologies of *BRCA1* mutation carriers were also not significantly different from those of non-carriers with respect to laterality, differentiation or histology.

Uncharacterized variants and frequent polymorphisms

A number of novel and previously reported uncharacterized variants were identified in the 107 cancer cases (Table 3). In addition, previously unreported silent base pair changes were found. These include the L^{87} (380G→A), T^{190} (689C→T), Q^{1401} (4322A→G) and H^{1862} (5705C→T) as well as a single heterozygous case of the previously reported D693N (2196G→A) polymorphism.

Table 3. Haplotypes containing uncharacterized variants

Number of haplotypes	Mutation	Amino acid change
2[a]	*798 G to A*	*Q227K*
1	2640 C to T	R841W
4	*4337 G to C*	*K1406N*
1	4654 G to T	S1512I
1	*5040 G to C*	*A1641P*
4	5193 G to A	D1692N
1	*5445 C to T*	*P1776S*
1	*5553 C to T*	*P1812S*

[a]Mutations indicated in italics have not been reported previously in the BIC database.

The subset of 91 non-Hispanic white cancer cases and 24 sister controls were screened for the 10 most common *BRCA1* polymorphisms using allele-specific amplification (ASA). These included the Q356R (1186A→G), S^{694} (2201C→T), L^{771} (2430T→C), P871L (2731T→C), G^{911} (2852A→G), M1008I (3143G→A), E1038G (3232A→G), K1183R (3667A→G), S^{1436} (4427C→T) and S1613G (4956A→G) polymorphisms. The G^{911} (2852A→G) and M1008I (3143G→A) polymorphisms were not detected in either population. Allele frequencies for the remaining eight common polymorphisms tested were similar to those reported by Dunning *et al.* (11) and Durocher *et al.* (12) (data not shown).

In our study, no significant differences were found in allele frequency or heterozygosity among the ovarian cancer cases and the sister controls. However, we found a trend towards an association between the rare form of the L871P polymorphism and ovarian cancer development. Durocher *et al.* (12) previously had reported a significant association ($P < 0.05$) between the rare form of the L871P polymorphism and breast/ovarian cancer incidence, where the P^{871} form occurred in a population-based control group at a frequency of 28% ($n = 113$) and in a familially unrelated consecutive series of cancer cases at a frequency of 42% ($n = 107$). Interestingly, the frequency of our matched sister controls and ovarian cancer cases showed a similar trend of 29% ($n = 24$) and 38% ($n = 24$), respectively, but the difference was not statistically significant.

The polymorphisms S^{694} (2201C→T), L^{771} (2430T→C), P871L (2731T→C), E1038G (3232A→G), K1183R (3667A→G), S^{1436} (4427C→T) and S1613G (4956A→G) were in pairwise linkage disequilibrium among the 46–48 chromosomes typed in our control population. The Q356R (1186A→G) polymorphism was not in linkage disequilibrium with the remaining seven polymorphisms (data not shown). There were no significant differences in the frequency of cancer cases with respect to the presence of at least one rare polymorphic allele and age at diagnosis (Table 4). However, there was a trend of older age at onset with the presence of the rare allele among the seven common polymorphisms except for the Q356R polymorphism.

Table 4. Distribution of common polymorphisms by age at diagnosis

Polymorphism	wt/wt			wt/pm			pm/pm		
	%[a]	Age (mean ± SD)	95% CI[b]	%	Age (mean ± SD)	95% CI	%	Age (mean ± SD)	95% CI
2201 C→T	58.9	55.7 ± 15.4	(52.5, 58.9)	31.1	57.8 ± 13.9	(54.9, 60.7)	10.0	63.0 ± 10.2	(60.9, 65.1)
2430 T→C	42.2	54.1 ± 16.1	(50.8, 57.4)	48.9	58.5 ± 13.4	(55.7, 61.3)	8.9	63.3 ± 10.7	(61.1, 65.5)
2731 C→T	39.6	53.5 ± 16.3	(50.2, 56.8)	41.8	59.1 ± 12.8	(56.5, 61.7)	18.7	60.4 ± 12.9	(57.7, 63.1)
3232 A→G	42.2	54.4 ± 15.9	(51.1, 57.7)	44.4	58.6 ± 14.3	(55.7, 61.5)	13.3	60.6 ± 9.5	(58.6, 62.6)
3667 A→G	38.2	54.9 ± 16.5	(51.5, 58.3)	49.4	58.2 ± 14.1	(55.3, 61.1)	12.4	60.7 ± 7.7	(59.1, 62.3)
4427 T→C	41.6	53.8 ± 15.3	(50.6, 57.0)	50.6	58.6 ± 14.4	(55.6, 61.6)	7.9	62.4 ± 6.0	(61.2, 63.6)
4956 A→G	42.2	54.0 ± 16.0	(50.7, 57.3)	44.4	58.5 ± 14.4	(55.5, 61.5)	13.3	61.8 ± 7.3	(60.3, 63.3)

wt, wild-type; pm, polymorphism.
[a]Percentage of cancer cases with the specified genotype at the locus indicated.
[b]95% confidence interval.

The rare 1186G allele was present relatively infrequently in this population, with an estimated allele frequency of 0.07 compared with the other polymorphisms with frequencies of 0.25–0.31. Comparison of the frequency of rare alleles among cases with a family history of cancer and sporadic cases showed that cases with a family history of ovarian cancer had a significantly higher frequency of the rare form (1186G) of the 1186A→G polymorphism (P = 0.03). Both probands with truncating mutations also had the 1186G polymorphism, one of whom was homozygous at this locus. There were no differences in the frequency of the remaining seven polymorphisms among cases with a family history of cancer compared with sporadic cases.

DISCUSSION

The frequency of truncating mutations in this series of ovarian cancer cases was 2/107 (1.9%). Both mutations occurred in the Caucasian population. This is the first estimate of the frequency of truncating *BRCA1* mutations in a population-based consecutive series of ovarian cancer cases. This estimate is similar to the frequency of *BRCA1* mutations (3.2%) detected in a large hospital-based consecutive-case series of 324 ovarian cancer patients not selected for a positive family history of cancer (13). It is lower than other published estimates in high risk ovarian cancer families (10/25 or 40%) (14). The *BRCA1* mutation frequency reported in another study of ovarian cancer cases not selected for a positive family history of breast or ovarian cancer was 8.6% (10/116) (15). This study, unlike ours and other published studies in ovarian cancer patients (13,14), was performed on primary ovarian tumors. Thus, a proportion of the *BRCA1* mutations detected may not have been germline, but rather somatic, in nature. Somatic *BRCA1* mutations in ovarian cancer have been reported (16), but the frequency of these mutations in the ovarian cancer population is not known.

BRCA1 mutation frequency estimates are also generally lower in studies of breast cancer patients not selected for a positive family cancer history compared with studies of sporadic cases. For example, Rebbeck *et al.* (17) reported that 14/23 (60.9%) of breast cancer patients from high risk families had *BRCA1* mutations. Couch *et al.* (5) reported that 16% of women with a family history of breast or ovarian cancer (n = 263) identified through a high risk clinic had *BRCA1* mutations. Krainer *et al.* (18) and Malone *et al.* (19) reported lower mutation frequencies of 12.3% (n = 73) and 6.2% (n = 193), respectively, in early-onset breast cancer patients not selected for family history of breast or ovarian cancer. Newman *et al.* (20) estimated the frequency of *BRCA1* mutations in a population-based study of breast cancer patients to be 3.3%.

It remains to be determined whether all *BRCA1* mutations were detected in this study. The methods of mutation detection that we used appear to be highly sensitive. Our laboratory has determined that the RNase mismatch cleavage assay was 93.3% efficient in detecting 70 out of 75 previously characterized mutations in the *p53* gene in lung tumors (unpublished data). The ASA test was 95.4% (124/130) efficient in detecting previously characterized *BRCA1* alterations in subjects with breast and ovarian cancer. One limitation of the *BRCA1* mutation detection methods in this study, which is common to most mutation screening techniques, is that they do not detect mutations affecting expression or stability of the protein, or deletions of large regions of the gene. These have been suggested to account for >30% of *BRCA1* mutations (21). Thus, the frequency of germline *BRCA1* mutations in this ovarian cancer population may be somewhat higher than reported.

Linkage studies suggest that >75% of breast/ovarian cancer families with three or more affected relatives have mutations in *BRCA1* (21,22), while 14% are associated with mutations in *BRCA2* (21). Mutations of the *BRCA2* gene have been suggested to be associated with an increased risk for ovarian cancer, albeit at a lower penetrance (23). However, we did not test for *BRCA2* mutations in our population, and the frequency of these mutations in this population-based ovarian cancer series is not known.

Several uncharacterized variants were identified in this study. Of these, three probands with variants in putative functional domains, R841W, Q227K and the A1641P alterations, had a family history of cancer. Our group previously has reported that the R841W alteration is associated with a family history of breast and ovarian cancer displaying a later disease onset (10). The R841W alteration is located in the putative RAD51-binding domain of *BRCA1*, and mutations in this region may alter RAD51-binding capacity and hence impair the cell's ability to execute DNA repair (24). In the present study, the proband carrying the R841W alteration had developed ovarian cancer at age >50 years. She also had one sister who had developed breast cancer at age >50 years.

The Q227K and A1641P alterations also occur within putative functional domains. The Q227K missense amino acid change occurs in a recently characterized p53-binding domain of BRCA1 (25). The proband carrying the Q227K alteration was homozygous for this change and had developed ovarian cancer at age >50 years and had a mother who had developed breast cancer at

age >50 years. The A1641P alteration is located in the transactivation domain of BRCA1 (26,27), adjacent to a BRCT repeat. In this family, the proband developed ovarian cancer at age <50 years and had a sister who developed breast cancer at age <50 years and a mother who developed breast cancer at age >50 years.

The remaining uncharacterized variants identified in this study, D1692N, P1776S and P1802S, also lie in putative *BRCA1* functional domains. However, no family history of cancer was reported among these cases. Functional assays will be required to determine whether any of these uncharacterized variants affect BRCA1 activity.

We found evidence that the presence of the rare form of the Q356R and the L871P polymorphisms may be associated with an increased risk for developing ovarian cancer. A significant association ($P = 0.03$) was observed between the rare form of the Q356R polymorphism and a positive family history of ovarian cancer. Our case–sister control data also support the previously suggested (12) positive association between the rare form of the L871P polymorphism and ovarian cancer incidence. Both the Q356R (25) and L871P (24) polymorphisms lie in putative functional domains of *BRCA1*. Further studies, including functional assays, will be required to determine the significance of these alterations.

In summary, this study is the first report of a comprehensive analysis of germline *BRCA1* alterations in a population-based, consecutive series of ovarian cancer patients. The frequency of alterations in this report reflects more accurately the frequencies in the general ovarian cancer population. The data presented suggest that some burden of hereditary ovarian cancer risk may be attributable to uncharacterized variants and possibly some rare polymorphisms of *BRCA1*. These data argue that a comprehensive analysis of *BRCA1* alterations should be performed in a larger series of ovarian cancer patients to determine the relative risk attributable to all types of *BRCA1* alterations.

MATERIALS AND METHODS

Subjects

Subjects were recruited through the Cancer Surveillance Program of Orange County (CSPOC). The CSPOC records all cancer cases from every hospital and health care facility in Orange County, CA, where the population base is ~2.5 million. The CSPOC is one of the regional registries of the California Cancer Reporting System, meeting all reporting requirements of the Surveillance, Epidemiology and End Results Program of the National Cancer Institute. Protocols for population-based ascertainment of ovarian cancer have been described previously (28). We applied rapid case ascertainment methods to identify and recruit cases to participate within 2 months of diagnosis. Physicians were notified that their patient(s) would be contacted regarding study participation. This was followed by a letter of introduction sent to the patient, and a telephone interview to determine any family history of cancer. Positive family history was defined as at least one first degree relative with breast cancer at <50 years of age or ovarian cancer at any age. Trained interviewers elicited information on types of cancer and dates of diagnoses, birth and death on all first and second degree relatives and first cousins of the proband, including both affected and unaffected family members. After signing a consent form, the proband completed an epidemiologic risk factor questionnaire and provided an 18 ml blood sample. All reported malignancies among family members were verified by obtaining pathology reports, and reviewing tumor tissue, clinical records and death certificates (if applicable).

Blood collection and processing

Buffy coat samples were prepared from fresh anti-coagulated blood and frozen at –80°C. RNA was extracted according to the method of Chomczynski and Sacchi (29) using the RNAgents kit (Promega, Madison, WI). Genomic DNA was isolated from leukocytes using a commercial kit (Qiagen, Valencia, CA).

BRCA1 cDNA synthesis was performed for exons 1a–10 and 12–24. Primers for these regions are listed in Table 5. Reverse transcription reactions were prepared with the *BRCA1*-specific primers 1007-6 and 823-3. This was followed by primary nested PCR with the addition of the respective paired primers, 1007-5 and 1007-1. The PCR conditions included 36 cycles of amplification; the annealing temperature of the first six cycles was 61°C, and 58°C for the following 30 cycles. Secondary nested PCR was carried out with primer pairs 1007-7 and 106-7 for the 5′ segment and 1007-3 and 823-4 for the 3′ segment. The secondary nested PCR was carried out for 30 cycles with an annealing temperature of 58°C. The 5′ product was 912 bp long and the 3′ product was 1765 bp long.

Table 5. Primers used for cDNA synthesis and RT–PCR of regions 5′ and 3′ of exon 11 of *BRCA1*

Protocol		Primers	Position[a]	Sequence
cDNA	5′ region	1007-6 (3′)	(19mer; 1521–1503)	5′-GGCTTAAGTTGGGGAGGCT-3′
	3′ region	823-3 (3′)	(22mer; [5711+203]–[5711+182][b])	5′-ATTTCCAAGGGAGACTTCAAGC-3′
First PCR	5′ region	1007-5 (5′)	(19mer; 1–19)	5′-AGCTCGCTGAGACTTCCTG-3′
		1007-6 (3′)		
	3′ region	1007-1 (5′)	(18mer; 4107–4124)	5′-AGCCAGGGAGTTGGTCTG-3′
		823-3 (3′)		
Nested PCR	5′ region	1007-7 (5′)	(16mer; 28–43)	5′-ACCAGGCTGTGGGGTT-3′
		106-7 (3′)	(18mer; 940–923)	5′-CATGGCTCCACATGCAAG-3′
	3′ region	1007-3 (5′)	(20mer; 4126–4145)	5′-GTGACAAGGAATTGGTTTCA-3′
		823-4 (3′)	(21mer; [5711+179]–[5711+158][c])	5′-AAAATCTTTAAGGGACCCTTG-3′

[a]The positions where the oligonucleotides bind to the *BRCA1* gene are indicated in parentheses, numbered in the 5′ to 3′ direction starting with the adenosine of the untranslated first exon as number +1 (according to the convention in BIC).
[b]Binds at the intron sequence 182–203 bp downstream of nucleotide 5711 (stop codon).
[c]Binds at the intron sequence 179–158 bp downstream of nucleotide 5711 (stop codon).

Table 6. PCR oligonucleotide primers used for RNase cleavage assay

Fragment	Region	Size (bp)	Primer[a]	Sequence[b]
5′B	Exons 2–7	421	BR1-5B-T7 (40mer; 95–108)	5′-CTGGGTAAAGTTCATTGG-3′
			BR1-5B-SP6 (40mer; 516–499)	5′-GGTTTCTGTAGCCCATAC-3′
5′C	Exons 6–11	530	BR1-5C-T7 (40mer; 390–407)	5′-TGTGCTTTTCAGCTTGAC-3′
			BR1-5C-SP6 (40mer; 920–903)	5′-TGAAACAGAACTACCCTG-3′
11A		1112	BR1-11A-T7 (41mer; [c])	5′-TCCACCTCCAAGGTGTATG-3′
			BR1-11A-SP6 (41mer; 1748–1730)	5′-ACCATTCTGCTCCGTTTGG-3′
11B		945	BR1-11B-T7 (45mer; 1666–1688)	5′-ATTTTATCAAGAAAGCAGATTTG-3′
			BR1-11B-SP6 (42mer; 2611–2592)	5′-TACTTAAAGCCTTCTGTGTC-3′
11C		887	BR1-11C-T7 (44mer; 2477–2498)	5′-GGAAGTTAGCACTCTAGGGAAG-3′
			BR1-11C-SP6 (43mer; 3364–3344)	5′-GCATTCAATTTTGGCCCTCTG-3′
11D		1093	BR1-11D-T7 (40mer; 3270–3287)	5′-ACTAATGAAGTGGGCTCC-3′
			BR1-11D-SP6 (40mer; [d])	5′-CACCTTAGGAGGAACATG-3′
3′C	Exons 11–16	849	BR1-3C-T7 (41mer; 4155–4173)	5′-GAAAGAGGAACGGGCTTGG-3′
			BR1-3C-SP6 (43mer; 5004–4983)	5′-CCATTGCATTATACCCAGCAG-3′
3′B	Exons 16–24	1015	BR1-3B-T7 (43mer; 4859–4879)	5′-TGAAGACAGAGCCCCAGAGTC-3′
			BR1-3B-SP6 (42mer; [e])	5′-CCTTGCATAGCCAGAAGTCC-3′

[a]The positions where the oligonucleotides bind to the *BRCA1* gene are indicated in parentheses, numbered in the 5′ to 3′ direction of the cDNA starting with the adenosine of the untranslated first exon as number +1.
[b]Sequences that bind to the DNA are listed. Sequences for T7/SP6 tails: T7 tails, 5′-GATAATACGACTCACTATAGGG-3′; SP6 tails, 5′-GCATTTAGGTGACAC-TATAGGA-3′.
[c]Binds at the intron sequence 110–154 bp upstream of exon 11.
[d]Binds at the intron sequence 148–131 bp downstream of exon 11.
[e]Binds 163–144 bp downstream of the stop codon.

Table 7. PCR conditions for ASA tests for common polymorphisms

Polymorphism	First round PCR			Second round PCR		
	F	R	Size[a] (bp)	NF	Annealing temperature (°C)	Size (bp)
1186 A→G	OM11AF	OM11BR	683	1186GF	60	204
2201 C→T	OM11EF	OM11FR	689	2201TF	60	376
2430 T→C	OM11EF	OM11FR	689	2430CF	60	147
2731 C→T	OM11GF	OM11HR	674	2731TF	65	420
2852 A→G	OM11GF	OM11HR	674	2852GF	65	299
3143 G→A	OM11HF	OM11JR	942	3143AF	61	585
3232 A→G	OM11HF	OM11JR	942	3232GF	61	496
3667 A→G	OM11HF	OM11JR	942	3667GF	63	61
4427 T→C	13F	13R	319	4427CF	63	141
4956 A→G	16F	16R	464	4956GF	67	215

F and R, 5′ and 3′ primers used for the first round PCR reactions, respectively, for each of the polymorphisms tested; NF, nested 5′ primer used in the second round PCR reaction.
[a]For each of the polymorphisms tested, the amplicon sizes for both the first and second round PCR reactions are indicated, as well as the annealing temperatures for the second round reactions.

RNase mismatch cleavage

DNA samples from the cancer cases were screened for mutations in the coding region of the *BRCA1* gene using the RNase Mismatch Cleavage Assay (NIRCA; Ambion, Austin, TX). The coding region was amplified in eight fragments (5′B, 5′C, 11A, 11B, 11C, 11D, 3′C, 3′B) using the primers listed in Table 6. Fragments 11A–11D were amplified from genomic DNA. The 5′ and 3′ fragments were amplified from cDNA.

The PCR mix contained (in a final volume of 15 µl), 1 µl of DNA (genomic 100 ng/µl stock; or 1/25 dilutions of the cDNA products described in the previous section), 0.45 µl of $MgCl_2$ (50

mM stock), 0.75 µl of 10× dNTP (12.5 mM dATP, dCTP, dGTP, dCTP each, stock), 1.5 µl of 10× PCR buffer (200 mM Tris–HCl pH 8.4, 500 mM KCl stock; Gibco, Grand Island, NY), 0.15 µl of T7 primer (50 pmol/µl stock), 0.15 µl of SP6 primer (50 pmol/µl stock) and 0.15 µl of *Taq* polymerase (5 U/µl stock; Gibco). The PCR program was 5 min at 94°C; 35 cycles of 45 s at 94°C, 1 min at 64°C (11A, 11B, 11C, 3′B, 3′C), 62°C (11D), 71°C (5′B), 65°C (5′C), 2 min at 72°C; followed by a 7 min extension at 72°C.

The PCR products then underwent *in vitro* transcription, as outlined in the kit, and cross-hybridization with *in vitro*-transcribed wild-type *BRCA1* derived from MCF7 cells. The double-stranded RNA then underwent incubation with a panel of three RNases at 37°C for 45 min. Cross-hybridized, double-stranded wild-type RNA served as the wild-type control. The digests were electrophoresed on 6% non-denaturing polyacrylamide gels and the gel fragments were visualized on an ultraviolet transilluminator following ethidium bromide staining. Fragments displaying digest patterns that were distinct from the wild-type patterns were sequenced using an ABI 377 automated sequencer. For truncating mutations and uncharacterized variants, we adopted the notation wt/wt, wt/m and m/m to denote the three genotypes at a particular locus.

ASA

DNA samples derived from the entire subset of 91 non-Hispanic white cases and 24 sister controls underwent screening for common polymorphisms using the ASA assay (30). The polymorphisms investigated are listed in Table 7. Among the 10 common polymorphisms, six result in a change of amino acid (1186A→G [Q356R], 2731C→T [P871L], 3143G→A [M1008I], 3232A→G [E1038G], 3667A→G [K1183R] and 4956A→G [S1613G]), while the remaining four are silent changes (2201C→T [S^{694}], 2430T→C [L^{771}], 2852A→G [G^{911}] and 4427C→T [S^{1436}]).

Table 8. PCR oligonucleotide primers used for ASA genotyping tests

Primer	Position[a]	Sequence[b]
OM11AF (5′)	(19mer; [790–82]–[790–64])	5′-CCACCTCCAAGGTGTATGA-3′
OM11BR (3′)	(21mer; 1390–1370)	5′-CCAGAATATTCATCTACCTCA-3′
OM11EF (5′)	(22mer; 1888–1903)	5′-GTATAAGCAATATGGAACTCGA-3′
OM11FR (3′)	(21mer; 2577–2557)	5′-TGGAACAACCATGAATTAGTC-3′
OM11GF (5′)	(20mer; 2477–2496)	5′-GGAAGTTAGCACTCTAGGGA-3′
OM11HR (3′)	(21mer; 3151–3131)	5′-TCAGGTGACATTGAATGTTCC-3′
OM11HF (5′)	(22mer; 2786–2807)	5′-TGGGTCCTTAAAGAAACAAAGT-3′
OM11JR (3′)	(20mer; 3728–3709)	5′-TCGGTAACCCTGAGCCAAAT-3′
EX 13F (5′)	(21mer; [4302–56]–[4302–36])	5′-AATGGAAAGCTTCTCAAAGTA-3′
EX 13R (3′)	(20mer; [4476+91]–[4476+72])	5′-TGTTGGAGCTAGGTCCTTAC-3′
EX 16F (5′)	(22mer; [4795–76]–[4795–55])	5′-AATTCTTAACAGAGACCAGAAC-3′
EX16R (3′)	(22mer; [5105+63]–[5105+42])	5′-AAAACTCTTTCCAGAATGTTGT-3′
1186A (wt)	(21mer; 1167–1187)	5′-AGAAAAGAATGGAATAAGC**A**G-3′
1186G (pm)	(21mer; 1167–1187)	5′-AGAAAAGAATGGAATAAGC**G**G-3′
2201C (wt)	(21mer; 2182–2202)	5′-CAAGTAAAAGACATGACAG**C**G-3′
2201T (pm)	(21mer; 2182–2202)	5′-CAAGTAAAAGACATGACAG**T**G-3′
2430T (wt)	(23mer; 2410–2432)	5′-TAGAGAGTAGCAGTATTTCA**T**TG-3′
2430C (pm)	(21mer; 2410–2430)	5′-TAGAGAGTAGCAGTATTTCA**C**-3′
2731C (wt)	(22mer; 2711–2732)	5′-AAAGCGCCAGTCATTTGCTC**C**G-3′
2731T (pm)	(22mer; 2711–2732)	5′-AAAGCGCCAGTCATTTGCTC**T**G-3′
2852A (wt)	(22mer; 2832–2855)	5′-AAAGGAAGAAAATCAAGGA**A**AG-3′
2852G (pm)	(22mer; 2831–2852)	5′-ACAAAAGGAAGAAAATCAAGG**G**-3′
3143G (wt)	(22mer; 3124–3145)	5′-ACTTTGAGGAACATTCAAT**G**TC-3′
3143A (pm)	(22mer; 3124–3145)	5′-ACTTTGAGGAACATTCAAT**A**TC-3′
3232A (wt)	(23mer; 3214–3236)	5′-GAGAAAATGTTTTTAAAG**A**AGCC-3′
3232G (pm)	(23mer; 3214–3236)	5′-GAGAAAATGTTTTTAAAG**G**AGCC-3′
3667A (wt)	(20mer; 3648–3667)	5′-TTTAGCAAAAGCGTCCAGA**A**-3′
3667G (pm)	(20mer; 3648–3667)	5′-TTTAGCAAAAGCGTCCAGA**G**-3′
4427T (wt)	(21mer; 4407–4427)	5′-CCTTCCATCATAAGTGACTC**T**-3′
4427C (pm)	(21mer; 4407–4427)	5′-CCTTCCATCATAAGTGACTC**C**-3′
4956A (wt)	(22mer; 4935–4956)	5′-AAAGTTGCAGAATCTGCCCAG**A**-3′
4956G (pm)	(22mer; 4935–4956)	5′-AAAGTTGCAGAATCTGCCCAG**G**-3′

wt, wild-type; pm, polymorphism.

[a]The positions where the oligonucleotides bind to the *BRCA1* gene are indicated in parentheses, numbered in the 5′ to 3′ direction starting with the adenosine of the untranslated first exon as number +1. Primer positions listed in square brackets indicate intron sequences.

[b]Nucleotides in bold indicate the specific base change for which the ASA tests were designed.

Genomic DNA samples underwent first round amplification as previously described (31) with the primers specified in Tables 3 and 4. The PCR program was 5 min at 94°C; 30 cycles of 45 s at 94°C, 1 min at 53°C, 2 min at 72°C; and a 7 min extension at 72°C. The product sizes of the first round amplification are listed in Table 7.

The PCR mix for the second round amplification contained 1 µl of the initial PCR product and involved nested forward primers for the respective tests (Tables 7 and 8). The PCR program was 5 min at 94°C; 13 cycles of 45 s at 94°C, 1 min at the specified annealing temperature; 1 min at 72°C; followed by a 7 min extension at 72°C. The product sizes are listed in Table 7. In order to test for heterozygosity, the first round amplification products underwent a second round PCR using a forward primer that incorporated a wild-type sequence at the locus of interest. The PCR conditions for the wild-type and polymorphism tests at each locus were identical. For the common polymorphism, we adopted the notation pm/pm to describe those homozygous for the less common allele at the particular locus and wt/pm to describe those heterozygous at that locus.

Statistical analyses

Differences among means of age at onset were determined using the Student's *t*-test, non-parametric Wilcoxon rank sum test and analysis of variance. The Hardy–Weinberg equilibrium was tested by χ^2 analysis where we compared the observed genotype frequencies with expected genotype frequencies under the Hardy–Weinberg equilibrium assuming the estimated allele frequencies. Pairwise linkage disequilibrium was assessed using likelihood methods (32). Because haplotypes of doubly heterozygous loci cannot be distinguished, we used the EM algorithm (33) in order to obtain maximum likelihood of haplotype frequencies and the disequilibrium coefficient. Associations between polymorphisms and/or uncharacterized variants and clinical characteristics among ovarian cases were tested by χ^2 analysis and Fisher's exact test. Associations between the presence of a sequence variant among cases and sister controls were estimated by conditional logistic regression (34).

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