ORIGINAL ARTICLE

Phenocopies in BRCA1 and BRCA2 families: evidence for modifier genes and implications for screening

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Classification of phenocopies and examples of pedigrees are available on http://jmg.bmjjournals.com/supplemental

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Background: The identification of *BRCA1* and *BRCA2* mutations in familial breast cancer kindreds allows genetic testing of at-risk relatives. Those who test negative are usually reassured and additional breast cancer surveillance is discontinued. However, we postulated that in high-risk families, such as those seen in clinical genetics centres, the risk of breast cancer might be influenced not only by the *BRCA1/BRCA2* mutation but also by modifier genes. One manifestation of this would be the presence of phenocopies in *BRCA1/BRCA2* kindreds.

Methods: 277 families with pathogenic *BRCA1/BRCA2* mutations were reviewed and 28 breast cancer phenocopies identified. The relative risk of breast cancer in those testing negative was assessed using incidence rates from our cancer registry based on local population.

Results: Phenocopies constituted up to 24% of tests on women with breast cancer after the identification of the mutation in the proband. The standardised incidence ratio for women who tested negative for the *BRCA1/BRCA2* family mutation was 5.3 for all relatives, 5.0 for all first-degree relatives (FDRs) and 3.2 (95% confidence interval 2.0 to 4.9) for FDRs in whose family all other cases of breast and ovarian cancer could be explained by the identified mutation. 13 of 107 (12.1%) FDRs with breast cancer and no unexplained family history tested negative.

Conclusion: In high-risk families, women who test negative for the familial *BRCA1/BRCA2* mutation have an increased risk of breast cancer consistent with genetic modifiers. In light of this, such women should still be considered for continued surveillance.

Epidemiological studies suggest that approximately 5% of breast cancer in Western countries is caused by high-risk dominantly inherited susceptibility genes.[1 2] However, twin studies suggested a genetic component in 27% of breast cancer cases.[3] High-risk dominantly inherited breast cancer susceptibility was first described in 1866 by Broca,[4] but proof of dominant inheritance was obtained 120 years later with the localisation and identification of *BRCA1* and *BRCA2*.[5–8] Mutations in these genes account for most high-risk families with ⩾4 breast cancers in members aged <60 years.[9] Studies to find other genes associated with breast cancer continue; the elusive *BRCA3* gene has not yet been identified.[10] In the mean time, many high-risk familial breast cancer kindreds are offered *BRCA1/BRCA2* mutation analysis. If this is successful, predictive genetic testing is offered to at-risk relatives. In some centres, relatives who test negative are reassured that their breast cancer risk is at population levels, as recommended by the recently published UK National Institute for Clinical Excellence (NICE) guidelines.[11] This policy assumes that, with few exceptions, the risk of a second high-risk familial mutation is minimal. However, breast cancer risks in high-risk kindreds with *BRCA1/BRCA2* mutations are substantially higher than risks derived from population-based studies.[9 12–14] In high-risk clusters in the Breast Cancer Linkage Consortium, *BRCA1* and *BRCA2* mutations were estimated to cause cumulative lifetime risks of breast cancer of 85–87% and 77–84% by 70 years, respectively.[9 15 16] However, estimates of breast cancer risks by 70 years from population-based studies are much lower (28–60%)[12–14] for *BRCA1* and still lower for *BRCA2*. It has been suggested that even these studies may overestimate the effect of *BRCA1/BRCA2* alone.[17] It is therefore feasible that a substantial proportion of the risk in familial clusters with a *BRCA1/BRCA2* mutation (the group of families that are usually seen by a Cancer Genetics Service) might be due to modifier genes.[18 19] Such a hypothesis would predict that some members of high-risk families might be at increased risk of breast cancer even if they tested negative for the pathogenic *BRCA1/BRCA2* mutation.[20] Phenocopies may be due to chance or environmental effects, another high-risk mutation in a family, ascertainment bias or the presence of modifier genes. We have identified a large number of phenocopies from 277 families with a pathogenic *BRCA1/BRCA2* mutation and have assessed breast cancer risks for people testing negative for a familial pathogenic mutation.

PATIENTS AND METHODS

Index cases and relatives

Families with breast or ovarian cancer have been tested for *BRCA1/BRCA2* mutations since 1996 in the overlapping regions of Manchester and Birmingham in mid-north England, covering around 10 million people. Women who attend the specialist genetic clinics in these two regions with a family history of breast or ovarian cancer have a detailed three-generation family tree elicited. If a *BRCA1/BRCA2* mutation is identified, further attempts are made to ensure that all people relevant to

Abbreviations: FDR, first-degree relative; NICE, National Institute for Clinical Excellence; SIR, standardised incidence ratio

discussions on risk are represented on the family tree. All cases of breast or abdominal cancer are confirmed by means of hospital or pathology records, Regional Cancer Registries (data available from 1960) or death certification. Once a family-specific pathogenic *BRCA1/BRCA2* mutation is identified, predictive testing is offered to all blood relatives. Where possible, all women with breast or ovarian cancer are tested to establish the true extent of *BRCA1/BRCA2* involvement in the family. In many instances, this entails obtaining paraffin-wax-embedded tumour block material from deceased relatives. In many large families, it is possible to establish ''obligate'' gene carriers by testing for the same mutation in different branches of the family, thereby establishing that intervening relatives carry the same mutation.

Details of all tested relatives and first-degree untested female relatives were entered on to a Filemaker Pro-5 database. The first person in whom a mutation was identified was designated the ''index'' case, with all other people being classified as to their position in the pedigree compared with a person who is a proved mutation carrier. All women reaching 30 years were entered into the study if untested for a mutation, as were all those with confirmed mutation status aged <30 years. The exception was the mother of a mutation carrier when the mutation was clearly paternally inherited. A total of 277 index cases were studied, and from these extended pedigrees information on 1444 women was collected. Date of birth and date of last follow-up, breast cancer status, ovarian cancer status, dates of diagnoses and date of death (if applicable), gene mutation carried in the family, the relationship with a known mutation carrier and the mutation status were recorded. The resultant combined series is known as the M6-ICE (Inherited Cancer in England) Study.

Patients with breast or ovarian cancer who tested negative for the family mutation were considered as phenocopies. A second blood sample was taken from every phenocopy and at least two techniques (sequencing, single-strand conformational polymorphism, protein truncation test) were used to establish the negative status. In addition, the mutation was confirmed by testing at least two samples from the index case or from another family member.

Classification of phenocopies

We developed a classification system (see supplementary information at http://jmg.bmjjournals.com/supplemental) to study the phenocopies. After an initial analysis, we concentrated on those people who were FDRs of a mutation carrier who unexpectedly tested negative for the family mutation despite there being no family history of breast cancer that is unaccounted for on either side of the family (designated type A_1 phenocopies).

Analyses

The relative risk of breast and ovarian cancers for different groups of women who tested negative was estimated by calculating a standardised incidence ratio (SIR; the ratio of the observed to expected number of cases).

Expected numbers were calculated using incidence rates for the period 1975–2004 from the North Western Cancer Registry. Age group, sex and calendar period-specific person-years at risk were multiplied by the corresponding incidence rates to produce the number of expected cases.

As follow-up began in 1975 (the earliest year for which reliable population-based incidence rates are available from the North Western Cancer Registry), women who were censored before 1 January 1975 were excluded from all analyses. Patients were censored at date of breast cancer diagnosis, prophylactic mastectomy, last follow-up or date of death, whichever was the earliest.

Relative risks were calculated for all relatives regardless of relationship with index cases, and for all first-degree relatives (FDRs; analysis 1).

A further analysis was undertaken on FDRs, excluding those with cases of breast or ovarian cancer in their families that could not be explained by the familial mutation, thus limiting the analysis to phenocopies A_1 only (analysis 2a).

To take account of testing bias (ie, FDRs with breast cancer more likely to be tested than those without) and to increase the power of the study, FDRs who were not tested were included, except those with ovarian cancer (analysis 2b). Untested relatives can be divided into those with breast cancer (untested affected) and those without (untested unaffected).

In unaffected women, the probability of testing negative increases with advancing age. Therefore, when estimating the proportion of untested unaffected relatives who would be expected to have been negative if tested, we divided women into three age groups, with different probabilities of being negative (see table 2). The numbers in each age group were multiplied by the probability of being negative and then added to obtain the estimated number that would have tested negative.

To estimate the number of untested affected FDRs who would have tested negative, the number of women in this group was multiplied by the proportion of those with breast cancer who tested negative (analysis 2a).

The observed and expected numbers of breast cancers for those who tested negative, for the unaffected untested and the affected untested were summated. The total number of observed cases was divided by the total number expected to provide the best estimate of the relative risk of breast cancer in FDRs with no unexplained cases in the family, who tested negative for the family mutation.

A further analysis was undertaken of the relative risk of women developing breast cancer who were not affected at the time of the negative test. The end point for this analysis was 31 December 2004 rather than the date of last follow-up.

An exact Poisson distribution was used to calculate 95% confidence intervals (CIs).

RESULTS

A total of 277 families with pathogenic *BRCA1/BRCA2* mutations were identified (165 *BRCA1* and 112 *BRCA2*): 190 index

Table 1 Distribution of tested and untested female first-degree relatives by mutation status regardless of censor dates

	Breast cancer	Ovarian cancer	Breast and ovarian cancer	Unaffected	Total
Index	190	48	33	6	277
FDR positive	84	11	11	142	248
FDR positive*	64	43	12	9	128
FDR negative	18	1	0	165	184
FDR unknown	127	57	13	215	412
Total	483	160	69	537	1249

FDR, first-degree relative.
*Positive mutation status inferred only after death by testing paraffin blocks (n = 19) or by identification as an intervening relative between two proved mutation carriers.

cases had breast cancer, 48 ovarian cancer, 33 both breast and ovarian cancer, and 6 were unaffected (table 1).

In all, 111 of 277 (40%) families contained at least one patient with ovarian cancer. Of 531 living female relatives who were tested, 258 (49%; age range 23–87 years; median 50 years) tested negative for the family mutation, including 28 with breast cancer (age range 31–71 years; median 49 years) and 4 with ovarian cancer (ages 60, 63, 64 and 66 years). Of all tests on living affected female relatives, 28 of 118 (24%) were negative.

Also, 184 of 560 (33%) FDRs on whom mutation status could be verified tested negative, including 13 type A_1 phenocopies (age range 31–71 years; median 45 years) and 5 other phenocopies (table 1). When this was confined to living people who had supplied a blood sample, the proportion testing negative rose to 184 of 432 (43%). Thus, 13 FDRs with no unexplained history of breast or ovarian cancer in the family who tested negative for the family mutation developed breast cancer; three were unaffected at the time of testing. In 11 of 13 families (4 *BRCA1*, 9 *BRCA2*) the initial affected proband had been examined for mutations in both genes (only a single mutation was found), and, in all 8 families with other living affected family members, the known mutation had been shown. In 12 of 13 type A_1 phenocopies, sufficient DNA was available for full gene testing, which excluded other mutations in *BRCA1* and *BRCA2*. Table 2 presents the ratio of those unaffected FDRs testing negative for the family mutation.

A prospective analysis was undertaken of all FDRs testing negative for the family mutation who were unaffected at the time of testing. Three breast cancers had occurred in 818 person-years of follow-up in 153 women, whereas only 1.4 were expected: SIR = 2.1 (95% CI 0.4 to 6.2). Notably, two of the other 10 type A_1 phenocopies occurred after the family was ascertained, but before a mutation was identified.

Analysis 1

In all relatives testing negative, irrespective of their relationship with a known mutation carrier, breast cancer developed in 28 of 258 (10.9%) women (table 3). The expected number of cancers from 1 January 1975 to the last follow-up in these women was 5.3, giving a relative risk of 5.3 (95% CI 3.5 to 7.7). When confined to FDRs, the risk was 5 (95% CI 2.9 to 7.8). We found 4 (1.6%) ovarian cancers occurring in the same population compared with 0.9 expected, giving an SIR of 4.6 (95% CI 1.2 to 11.7).

Analysis 2

Figure 1 shows how the FDRs included in analysis 2 were chosen. In all, 13 of 107 (12.1%) FDRs with breast cancer tested negative (type A_1 phenocopy). The proportion of living patients with breast cancer testing negative in this situation was 13 of 87 (15%). The relative risk in 166 women who tested negative was 4 (95% CI 2.1 to 6.9).

Sixty three women who were not tested and who had not been censored before 1 January 1975 developed breast cancer. If we assume the lower estimate of these being mutation negative as in the identified group (12.1%), the estimate for the observed number of cancers in this group would be 7.7, giving a total observed number of cases of 20.7. Of the 188 FDRs who were not tested and who did not develop breast cancer, 119 were expected to be negative, if tested. The expected number of breast cancers for the tested negative, untested affected and untested unaffected combined is 6.4, giving a relative risk of 3.2 (95% CI 2.0 to 4.9). Even in the unlikely instance that none of the 63 untested breast cancers was a phenocopy, the relative risk of breast cancer would still be >2.

Table 2 Proportion of unaffected first-degree relatives of mutation carriers testing negative for the family mutation depending on age

Age at testing (years)	Testing positive	Testing negative	Proportion negative (%)	Assumed proportion negative (%)
18–39	75	62	45	50
40–59	51	73	59	60
⩾60	4	26	87	90
Total	130	161		

The table does not include 17 unaffected female first-degree relatives who tested positive (or were obligate carriers) but had a daughter with breast or ovarian cancer who also tested positive.

DISCUSSION

Phenocopies in diseases other than *BRCA1/BRCA2* have been well described. Three Southern Chinese phenocopies with endocrine tumours were reported as multiple endocrine neoplasia type 1.[21] In all, 10% of patients with multiple endocrine neoplasia type 1 diagnosed with an unspecific clinical investigation and not by genetic analysis were phenocopies.[22] Several patients with apparent Huntington's disease did not have a mutation, but had a mutation in another gene.[23] Finally, a phenocopy of CAII deficiency was, when investigated, found to be due to coinheritance of two separate mutations.[24]

We identified four different categories of phenocopy for *BRCA1/BRCA2*. Although some phenocopies will have occurred by chance, we have shown that this does not account for the excess of breast cancers observed both by the early median age at diagnosis and by the increased relative risk. Our analysis of relative risk has controlled for the potential testing bias and allowed for the maximum possible number of unaffected relatives being negative for the family mutation. Even in a prospective analysis, where we cannot be sure we would have been informed of all breast cancers, the relative risk for those FDRs testing negative is twofold. Although the proportion of FDRs for which the mutation status could be validated as negative was only 33%, this rose to 43% when we confined this to living people. In analysis 2, when we included untested unaffected relatives, the ratio of assumed positive to negative reached the expected 50:50. We also used a conservative estimate for the proportion of untested affected FDRs with breast cancer who would have tested negative.

Double heterozygotes (women with a mutation in both *BRCA1* and *BRCA2*) have been reported in both Ashkenazi Jewish families and Caucasian pedigrees.[25–29] Phenocopies may be due to the existence of a second, unidentified, *BRCA1/BRCA2* mutation. However, in our study, 12 of 13 phenocopies have tested negative for both genes. Additionally, 10 000 consecutive *BRCA1/BRCA2* samples showed only 11 double heterozygotes, all of Ashkenazi Jewish origin.[30] Therefore, we hypothesise that the increased risk is due to the presence of other susceptibility genes in tested families.

Antoniou *et al*[10] postulated that either a recessive *BRCA3* gene with a penetrance of around 42% existed or a polygenic model was the answer. The polygenic model hypothesised modifying genes that increased the effect on both *BRCA* mutation-positive people and those without a *BRCA* mutation. Rebbeck[31] suggested that genes associated with the metabolism of environmental carcinogens and those associated with steroid hormone metabolism could modify *BRCA1/BRCA2*-associated breast cancer risk. Therefore, if a family had a *BRCA* mutation along with mutations of modifier genes, then a *BRCA*-negative woman could still inherit a susceptibility allele (or alleles) for

Table 3 Risk of breast cancer in relatives who tested negative for mutation

Group of relatives	Number	Number of observed cases	Number of expected cases	RR (95% CI)
Relatives regardless of family history (analysis 1)				
All relatives	257	28	5.3	5.3 (3.5 to 7.7)
FDRs only	184	18	3.6	5.0 (2.9 to 7.8)
FDRs with no unexplained cases in family (analysis 2)				4.0 (2.1 to 6.9)
Tested negative	166	13	3.2	
Untested affected	63	7.7 (12% of 63)	0.1	
Untested unaffected	188	0	3.1	
Total		20.7	6.4	3.2 (2.0 to 4.9)

FDR, first-degree relative.

developing early-onset breast cancer. Reports of families where *BRCA1/BRCA2* mutations could not be identified but still several breast cancers had been found[32] were attributed to a mutation in the RAD51D gene with which both BRCA proteins interact. *BRCA2* carriers were put at an increased risk of breast cancer if they carried not only their *BRCA2* mutation but also a RAD51 mutation.[19] This effect was not found for *BRCA1* or non-mutation carriers. Therefore, evidence regarding modifier genes for breast cancer does exist, although few studies have been validated.[33]

Modifier genes are likely to be more common in women who are tested for mutations in a Clinical Cancer Genetics Service setting than in the general population. Thus, the penetrance in our BRCA1/2 Clinical Cancer Genetics Service families is similar to that in the Breast Cancer Linkage Consortium[15 16 34] families (penetrance by 70 years of 80–90% (data not shown)). This is much higher than that in population-based samples and particularly in families ascertained through Fanconi anaemia.[35] Penetrance estimates from our families would support a 2–3-fold higher penetrance to age 70 years than in families not ascertained on the basis of multiple early-onset cases of breast cancer. Interestingly, a recent analysis of penetrance of the Icelandic *BRCA2* founder mutation calculated that the risk for relatives testing negative for the mutation was 10.7% at age 70 years rather than 7.5% in the general population.[36] This relative risk of 1.43 was present despite the population-based aspect of this study. Indeed, the relative risk at younger ages is likely to have been higher. The relative risk of 3.2 in our study was for 50 years of age and probably reflects only a doubling of lifetime risk. Despite our efforts to correct for testing bias, the process of selecting families for testing may have exaggerated the risks for those testing negative. Only long-term prospective studies will fully answer this question. Although the implications of these results in less highly selected families may be less, they should be relevant to most families attending Clinical Cancer Genetics services worldwide.

This study has shown that the breast cancer risk of a female FDR of a known *BRCA1/BRCA2* mutation carrier who tests negative for the familial mutation is 6.4% by the age of 50 years. The risk of the general population is approximately 2% by this age. If confirmed, these findings have serious implications for the management of these people. The recently published US National Comprehensive Cancer Network guidelines for breast screening recommend that women who have a 5-year risk of invasive breast cancer of >1.7% be offered annual mammograms from the age of 35 years.[37] Thus, women who test negative for familial *BRCA1/BRCA2* mutations would fulfil these criteria, and we propose that they should be considered for breast screening from the age of 35 years as per these recommendations in North America. At present, the UK NICE guidelines state that ''a woman who tests negative for a family mutation is at population risk'',[11] and these women are generally removed from their screening programmes and reassured. The NICE threshold for early screening (3% risk between 40 and 49 years) applies to women testing negative who would meet these criteria for ''moderate'' risk and qualify for mammography aged 40 rather than 50 years, which is the starting point in the general population in the UK. Clearly, there is a need for a prospective study of breast cancer incidence in those women testing negative to inform decisions about ongoing surveillance.

Although shared environmental or hormonal and reproductive factors could contribute to the increase in risk, environmental factors in particular are not a likely cause as they account for only about 10% of familial clustering.[38 39] Age at first pregnancy and parity, which have a marked influence on risk, did not seem to be a factor in our population. Newly identified risk factors, such as mammographic density, that increase breast cancer risk by up to threefold even among BRCA carriers[40] could be a candidate marker for the risks identified in our analysis, and there is some evidence that these could be heritable. If the reasons for the increased risk are polygenic or

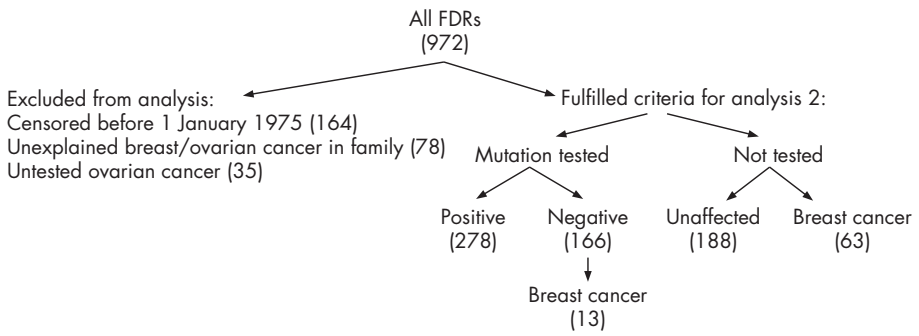

Figure 1 First-degree relatives (FDRs) who were included in analysis 2.

multifactorial shared factors, these are likely to be a relevant factor only for FDRs.

Finally, this study suggests that if a *BRCA1/BRCA2* mutation is not identified in an affected person with a suspected family history of breast or ovarian cancer, mutation analysis should be carried out on another affected relative. The index case may be a phenocopy, and further information on the family should be sought, including clarification of the breast pathology.[41] Ideally, an affected relative in the middle of the cluster (with an affected daughter) should be chosen rather than a young patient with breast cancer on the edge of the cluster. Unfortunate or inappropriate selection of an affected relative could cause a reduction in sensitivity of mutation testing of 6%, which is close to the missing value when adding strategies such as multiplex ligation-dependent probe amplification and direct sequencing.[42] However, our results do not justify full mutation testing of *BRCA1/BRCA2* in those undertaking predictive genetic testing for a known family mutation.

CONCLUSION

This study shows that *BRCA1/BRCA2* phenocopies pose an important clinical problem for the optimum management of families with familial breast cancer. In addition to causing false-negative *BRCA1/BRCA2* mutation analysis results, the increased frequency of phenocopy cases in *BRCA1/BRCA2* families provides evidence for the effect of modifier genes in highly penetrant families. Clinical cancer genetics clinics should attempt to verify the *BRCA1/BRCA2* mutation status of as many affected relatives as possible, as the daughters of phenocopies are likely to have considerably less risk than a *BRCA1/BRCA2* carrier. However, our findings suggest that even if all cancer cases are explained by the family mutation, women testing negative may remain at some increased risk and should be considered for continuing surveillance.

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