A single nucleotide polymorphism in the *RAD51* gene modifies cancer risk in *BRCA2* but not *BRCA1* carriers

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***BRCA1* and *BRCA2* carriers are at increased risk for both breast and ovarian cancer, but estimates of lifetime risk vary widely, suggesting their penetrance is modified by other genetic and/or environmental factors. The BRCA1 and BRCA2 proteins function in DNA repair in conjunction with RAD51. A preliminary report suggested that a single nucleotide polymorphism in the 5′ untranslated region of *RAD51* (135C/G) increases breast cancer risk in *BRCA1* and *BRCA2* carriers. To investigate this effect we studied 257 female Ashkenazi Jewish carriers of one of the common *BRCA1* (*185delAG*, *5382insC*) or *BRCA2* (*6174delT*) mutations. Of this group, 164 were affected with breast and/or ovarian cancer and 93 were unaffected. *RAD51* genotyping was performed on all subjects. Among *BRCA1* carriers, *RAD51-135C* frequency was similar in healthy and affected women [6.1% (3 of 49) and 9.9% (12 of 121), respectively], and RAD-135C did not influence age of cancer diagnosis [Hazard ratio (HR) = 1.18 for disease in *RAD51-135C* heterozygotes, not significant]. However, in *BRCA2* carriers, *RAD51-135C* heterozygote frequency in affected women was 17.4% (8 of 46) compared with 4.9% (2 of 41) in unaffected women ($P = 0.07$). Survival analysis in *BRCA2* carriers showed *RAD51-135C* increased risk of breast and/or ovarian cancer with an HR of 4.0 [95% confidence interval 1.6–9.8, $P = 0.003$]. This effect was largely due to increased breast cancer risk with an HR of 3.46 (95% confidence interval 1.3–9.2, $P = 0.01$) for breast cancer in *BRCA2* carriers who were *RAD51-135C* heterozygotes. *RAD51* status did not affect ovarian cancer risk. These results show *RAD51-135C* is a clinically significant modifier of *BRCA2* penetrance, specifically in raising breast cancer risk at younger ages.**

Germ-line mutations in the *BRCA1* and *BRCA2* genes increase susceptibility for both breast and ovarian cancer. Penetrance of these mutations is incomplete and age-dependent, thus cancer risk in carriers continues to increase with age even though the mean age of cancer diagnosis is younger in *BRCA1/BRCA2* carriers compared with noncarriers (1). Estimates of penetrance have varied widely, perhaps as a result of different ascertainment schemes and/or allelic effects. In families ascertained for multiple affected individuals suitable for linkage analysis, lifetime cancer risk (by age 70) was 85% for breast cancer in both *BRCA1* and *BRCA2* carriers (2, 3), 63% for ovarian cancer in *BRCA1* carriers (2), and 27% for ovarian cancer in *BRCA2* carriers (3). Significantly lower risk estimates were obtained in studies performed in less selected families or at the population level, with a 36–56% lifetime risk for breast cancer (4–7) and a 16% lifetime risk for ovarian cancer (5). These studies were performed in specific ethnic groups (Ashkenazi Jewish and the Iceland population) that harbor a limited number of specific mutations and could therefore be representative of these alleles, rather than reflect the general penetrance of *BRCA1/BRCA2* mutations. Such differences suggest that penetrance of *BRCA1/BRCA2* mutations is modified by other genetic and/or environmental factors. Identification of such modifiers has important implications, e.g., in facilitating more accurate risk assessment in carriers who face difficult clinical choices regarding prophylactic mastectomy and oophorectomy.

Candidate modifiers include genes whose products are known to interact with BRCA1 and BRCA2 (reviewed in ref. 8). RAD51 is a homologue of bacterial RecA, which is required for meiotic and mitotic recombination and for recombinational repair of double-strand DNA breaks. Both BRCA1 and BRCA2 have been shown to interact with RAD51 (9–11), and the phenotype of murine Brca1- and Brca2-knockout mice is similar to that of *Rad51* knockouts (reviewed in ref. 8). A missense mutation in *RAD51* (Arg-150–Glu) has been described in two Japanese patients with bilateral breast cancer (12), and Wang *et al.* orally presented evidence that a single nucleotide polymorphism (SNP) in the 5′ untranslated region (UTR) of RAD 51 is associated with increased breast cancer risk in *BRCA1* and *BRCA2* carriers but does not influence breast cancer risk in women who are not *BRCA1/BRCA2* carriers.‖ This SNP, designated 135 g/c, is a substitution of G for C at position 135 of the human RAD51 cDNA (GenBank accession no. D14134). The aim of the present study was to investigate further the association between the *RAD51* 5′ UTR polymorphism and disease status in *BRCA1* and *BRCA2* carriers and to determine whether the RAD51-135C polymorphism is indeed a modifier of *BRCA1* and/or *BRCA2* penetrance.

Methods

Subjects and Clinical Data. Participants included all 289 female Ashkenazi Jewish carriers, both healthy and affected, ascertained through Cancer Genetics clinics at two institutions in Israel: Shaare Zedek Medical Center (SZMC) in Jerusalem and Rambam Medical Center (RMC) in Haifa. Nineteen carriers at SZMC were identified through an ongoing study of all Ashkenazi Jewish women diagnosed with breast or ovarian cancer at SZMC since January 1995. All other carriers were identified through patients counseled for family history of breast and/or ovarian cancer history. Clinical data collected on each subject included type of malignancy (based on pathology reports), age at diagnosis or at last follow-up exam, and age at prophylactic surgery if any was performed. All women received genetic counseling and gave informed consent for genetic testing. The study was approved by the institutional review boards (Helsinki commit-

Abbreviations: HR, Hazard ratio; NS, not significant; CI, confidence interval.

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Table 1. *RAD51-135C* frequency in *BRCA1* and *BRCA2* mutation carriers

Disease status	*BRCA1* carriers		*BRCA2* carriers	
	RAD51-135C frequency	Mean age*, SD	*RAD51-135C* frequency**	Mean age*, SD
Healthy	3/49 (6.1%)	47.8 (13.5)	2/41 (4.9%)	45.6 (10.7)
Total affected	12/121 (9.9%)	44.9 (10.3)	8/46 (17.4%)	49.9 (10.9)
Unilateral breast cancer	6/64 (9.4%)	43.0 (10.6)	5/28 (17.9%)	46.0 (9.2)
Bilateral breast cancer	0/14 (0%)	41.9 (7.8)	1/5 (20%)	45.6 (10.4)
Breast & ovarian cancer	3/13 (23%)	50.4 (7.4)	1/1 (100%)	62
Ovarian cancer	3/30 (10%)	48.0 (10.6)	1/12 (8.3%)	61.2 (7.3)

*Mean age was not significantly different between affected and unaffected *BRCA1* carriers. In *BRCA2* carriers, $P = 0.07$.
**In *BRCA2* carriers $\chi^2 = 11.1$ for *RAD51-135C* frequency and disease status (4 df), $P = 0.03$. For RAD51-135C frequency in healthy vs. affected carriers, $P = 0.07$.

tees) at both SZMC and RMC. Samples from healthy Ashkenazi controls (SZMC) were anonymous DNA samples from unrelated healthy persons who reported all four grandparents as being Ashkenazi Jewish and who gave their consent for anonymous testing.

Molecular Analysis. *DNA extraction.* Genomic DNA was extracted from peripheral blood samples by using standard high-salt extraction (13).

Analysis of BRCA1 *and* BRCA2 *mutations.* Genomic DNA was analyzed for the three founder mutations common in Ashkenazi Jews (*BRCA1–185delAG, 5382insC,* and *BRCA2–6174delT*) by using previously published methods (4, 14).

RAD51 *genotyping. RAD51* genotyping was performed by PCR amplification of a 157-bp region around nucleotide 135. This amplicon contains a single *Mva*I site that is abolished by the 135C polymorphism. Wild-type alleles are digested by *Mva*I resulting in 86- and 71-bp products. The *135C* allele is not digested by *Mva*I, resulting in a single 157-bp product. PCR was performed by using the following primers: RAD51AF (5′-TGGGAACTGCAACTCATCTGG-3′) and RAD51RR (5′-GCGCTCCTCTCTCCAGCAG-3′) at a final Mg concentration of 1.5 mM and an annealing temperature of 53°C. After digestion with *Mva*I (Fermentas, Vilnius, Lithuania) for 4 h at 37°C, samples were run on a 3% agarose gel. Direct sequencing of the RAD51 amplicon (ABIprism 377, Perkin–Elmer) was performed on three samples heterozygous for the *Mva*I site, confirming the accuracy of the restriction-digest assay in identifying the *RAD51-135C* polymorphism.

Statistical Analysis. *RAD51* allele frequencies in affected vs. unaffected subjects were compared by using the χ^2 test. The association of disease status in *BRCA1/BRCA2* carriers and *RAD51-135C* was analyzed by using logistic regression, and analysis of disease-free survival was done by using Cox proportional hazard. Several outcomes were analyzed–breast and/or ovarian cancer, breast cancer only, and ovarian cancer only. In all analyses, healthy carriers were censored at the age of last follow-up exam or at the age of the relevant prophylactic surgery (i.e., prophylactic oophorectomy for ovarian cancer and prophylactic mastectomy for breast cancer). Outcomes in affected women were treated as follows: (*i*) for analysis of breast and/or ovarian cancer, outcome in affected women was the age at diagnosis of the first malignancy; (*ii*) for analysis of breast cancer only, the outcome in women affected with breast cancer was the age of breast cancer diagnosis; women with ovarian cancer were censored at the age of prophylactic mastectomy, last follow-up exam, or death (whichever came first); and (*iii*) for analysis of ovarian cancer only, the outcome in women affected with ovarian cancer was the age of ovarian cancer diagnosis, and women with breast cancer were censored at age of prophylactic oophorectomy, last follow-up exam, or death (whichever came first).

Results

There are 289 *BRCA1* and *BRCA2* carriers currently followed at both participating institutions. *RAD51* mutation analysis could not be performed in 25 cases, and the age at diagnosis was not known in three cases, leaving a total of 261 subjects in which complete clinical data and genetic analyses were available. Two subjects were excluded because they were affected with cancers other than breast or ovarian (one with colorectal cancer and one with lymphoma). Two additional subjects were excluded because they were double heterozygotes (*BRCA1–185delAG* and *BRCA2-6174delT*) and could not be assigned a single mutation status. Analysis therefore was performed on 257 carriers who are members of 205 unrelated families (141 segregating *BRCA1* mutations and 64 segregating *BRCA2* mutations).

Among all carriers, 93 were unaffected, and 164 were affected with breast and/or ovarian cancer. Of 170 *BRCA1* carriers, 49 (29%) were unaffected, and of 87 *BRCA2* carriers, 41 (47%) were unaffected. Mean age at diagnosis in affected carriers and mean age at last follow-up exam in healthy carriers were not significantly different (Table 1). No *RAD51-135C* homozygotes were identified among all carriers or among 73 healthy Ashkenazi controls. *RAD51-135C* heterozygote frequency among all healthy carriers was 5.5% (5 of 90) compared with 12% (20 of 167) among affected carriers. This difference was not significant (Table 1). In *BRCA2* carriers, there was a trend to higher frequency of *RAD51-135C* among affected carriers ($P = 0.07$). *RAD51-135C* heterozygote frequency in 73 healthy Ashkenazi controls was 8.2% (6 of 73), intermediate between the frequencies observed in affected and unaffected *BRCA1/BRCA2* carriers.

Initial analysis was performed only for unrelated cases. Results were similar to those presented below for all cases, but power was obviously lower because of the smaller sample size. Except for one mother/daughter pair, both of which were *BRCA1* carriers and *RAD51-135C* heterozygotes, all other *RAD51-135C* heterozygotes were unrelated to each other. Furthermore, *RAD51*, *BRCA1*, and *BRCA2* are located on different chromosomes and segregate independently, and thus information gained from multiple members of the same family is pertinent to the question of genetic interaction between *BRCA1/BRCA2* and *RAD51*. Results therefore are presented for all cases (257 carriers from 205 families). We first analyzed the effect of *RAD51-135C* on diagnosis of any related malignancy (breast and/or ovarian cancer) in all carriers (*BRCA1* and *BRCA2* combined). *RAD51* status did not influence total malignancy risk [Hazard ratio (HR) = 1.46 for *RAD51-135C* vs. normal homozygotes, not significant (NS)]. However, as a covariate, the specific *BRCA* gene was a significant predictor of disease risk [HR = 0.6,

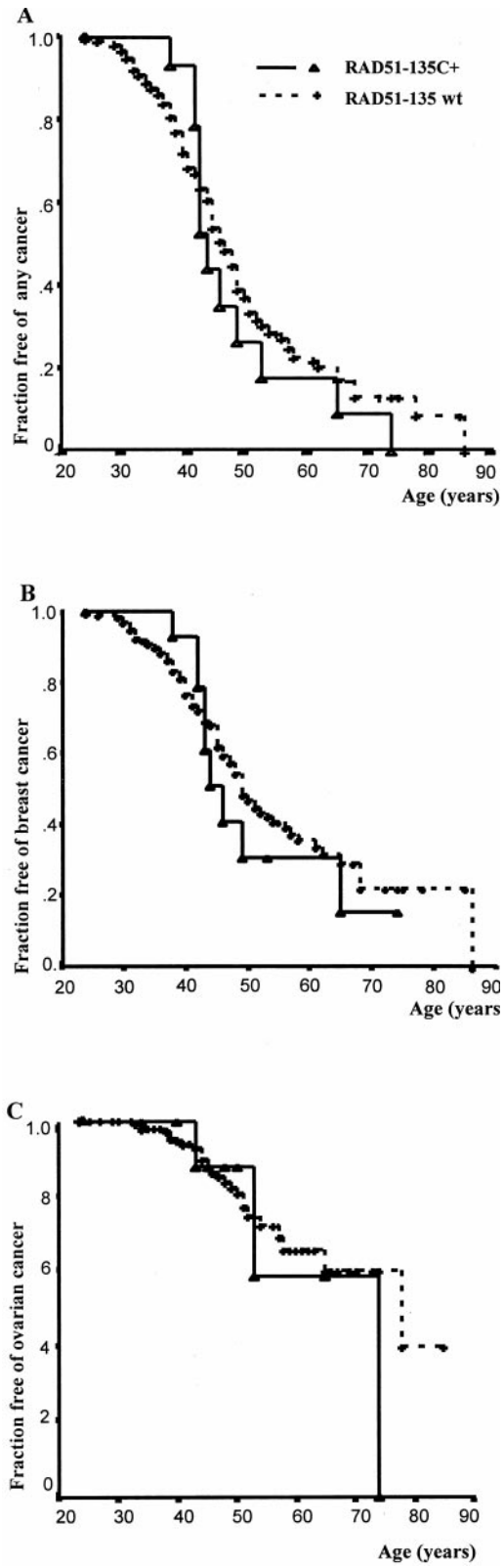

Fig. 1. Cancer risk in *BRCA1* carriers–survival analysis according to *RAD51* genotype. RAD51-135C +- heterozygotes for the *RAD51-135C* polymorphism. RAD51-135wt (wild type) − homozygous normal (GG) at nucleotide 135. (*A*) Fraction of *BRCA1* carriers remaining free of both breast and ovarian cancer. For *RAD51-135C* heterozygotes, HR = 1.18 (NS). (*B*) Fraction of *BRCA1* carriers remaining free of breast cancer. For *RAD51-135C* heterozygotes, HR = 1.16 (NS). (*C*) Fraction of *BRCA1* carriers remaining free of ovarian cancer. For *RAD51-135C* heterozygotes, HR = 1.28 (NS).

$P = 0.01$ for *BRCA2* vs. *BRCA1*], and thus further analyses were performed separately for *BRCA1* and *BRCA2* carriers. This separate analysis is justified also on the grounds that any gene–gene interaction would not necessarily be similar for both *BRCA1* and *BRCA2*.

In *BRCA1* carriers, *RAD51-135C* was not found to influence disease risk (Fig. 1). This lack of effect was true for both breast and ovarian cancer combined (HR = 1.18, NS; Fig. 1*A*), breast cancer only (HR = 1.16, NS; Fig. 1*B*), and ovarian cancer only (HR = 1.28, NS; Fig. 1*C*). However in *BRCA2* carriers, *RAD51-135C* significantly increased cancer risk (Fig. 2). For both breast and ovarian cancer combined, the HR for *BRCA2* carriers who were also *RAD51-135C* heterozygotes was 4.0 (95% CI 1.6–9.8, $P = 0.003$; Fig. 2*A*). As noted above, information gained from multiple family members is relevant to genetic interaction between independently segregating loci. However, if a substantial proportion of *RAD51-135C* heterozygotes among *BRCA2* carriers were related closely, other unidentified familial factors could have confounded the observed *RAD51–BRCA2* interaction. To exclude this possible bias rigorously (even though all *RAD51-135C* heterozygotes among *BRCA2* carriers were unrelated), analysis in *BRCA2* carriers was performed also by using only unrelated subjects. In unrelated *BRCA2* carriers, for both breast and ovarian cancer combined, the HR for *RAD51-135C* heterozygotes was 3.5 (95% CI 1.4–8.9, $P = 0.008$). Notably, all *BRCA2/RAD51-135C* carriers became affected by age 58, whereas among *BRCA2* carriers who were not *RAD51-135C* heterozygotes, 50.4% remained unaffected at the same age.

To explore the *RAD51–BRCA2* interaction further, it also was tested for breast and ovarian cancer as separate outcomes. *RAD51-135C* did not influence ovarian cancer risk in *BRCA2* carriers [HR = 1.23 (NS), $P = 0.85$ for *RAD51-135C* heterozygotes vs. normal homozygotes], but the number of ovarian cancer outcomes in *BRCA2* carriers was small (Fig. 2*C*). Breast cancer risk, however, was elevated significantly in *BRCA2* carriers who were also *RAD51-135C* heterozygotes with an HR of 3.46 (95% CI 1.3–9.2, $P = 0.01$; Fig. 2*B*). Thus, most of the elevated cancer risk associated with the *RAD51-135C* polymorphism in *BRCA2* carriers is explained by an increase in breast cancer risk. Similar results were obtained with analysis by logistic regression, in which the outcome is dichotomous (presence or absence of breast cancer diagnosis, regardless of age at diagnosis). In *BRCA1* carriers, breast cancer risk was not elevated significantly in *RAD51-135C* heterozygotes (odds ratio = 1.1, NS), but in *BRCA2* carriers, the odds ratio for breast cancer in *RAD51-135C* heterozygotes was 4.3 ($P = 0.046$). Notably, the magnitude of the *RAD51-135C* effect found by logistic regression was similar to that found with survival analysis by using Cox proportional hazard. The level of significance is lower because of reduced power when information from age at diagnosis is not taken into account.

Discussion

We observed that in *BRCA2–6174delT* carriers, presence of the *RAD51-135C* allele results in an approximately 4-fold increase in breast cancer risk (Fig. 2). This elevated risk was observed consistently by using different analyses (Cox proportional hazard and logistic regression) and in independent unrelated cases as well as in the entire study group. *RAD51-135C* did not influence breast or ovarian cancer risk in *BRCA1–185delAG/5382insC* carriers (Fig. 1). Because both genetic and environmental modifiers are more likely to influence low-penetrance mutations than high-penetrance mutations, these results are consistent with previous observations of lower penetrance of *BRCA2* and more specifically the *BRCA2–6174delT* mutation. As noted above, studies in high-risk families found ovarian cancer risk is lower in *BRCA2* carriers compared with *BRCA1* carriers (3), and despite similar life-

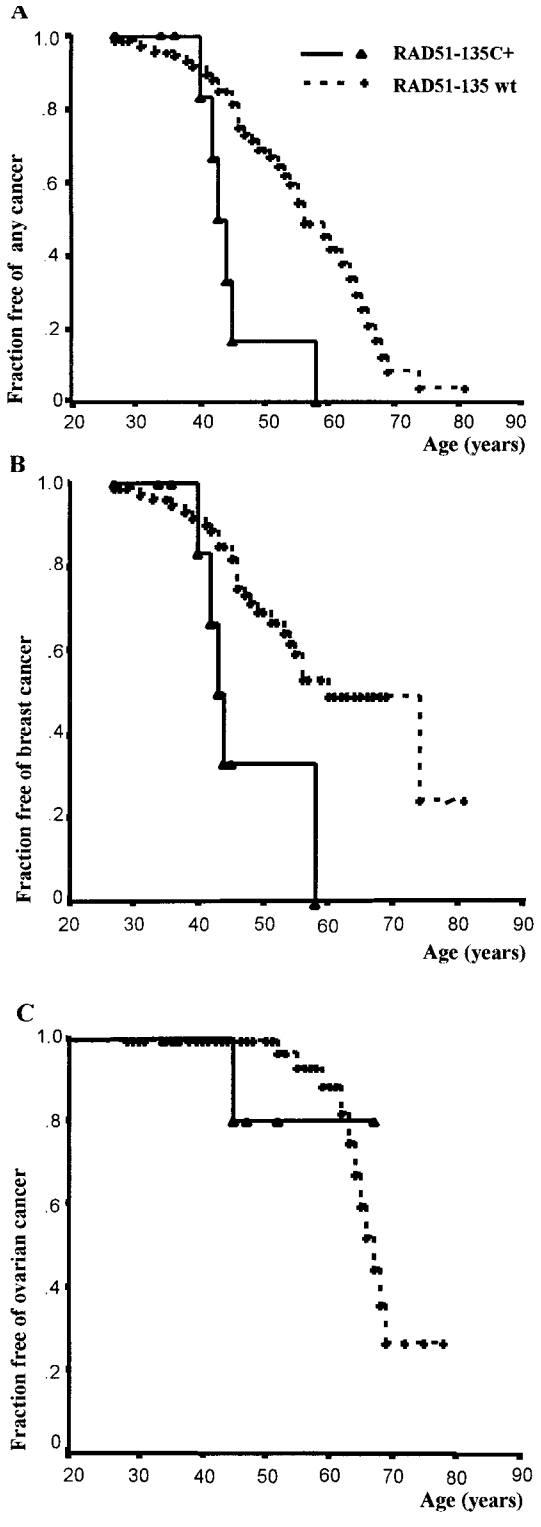

Fig. 2. Cancer risk in *BRCA2* carriers–survival analysis according to *RAD51* genotype. RAD51-135C +-heterozygotes for the 135C polymorphism. RAD51-135wt (wild type) − homozygous normal (GG) at nucleotide 135. (*A*) Fraction of *BRCA2* carriers remaining free of both breast and ovarian cancer. For *RAD51-135C* heterozygotes, HR = 4.0 [95% confidence interval (CI) 1.6–9.8, $P = 0.003$]. (*B*) Fraction of *BRCA2* carriers remaining free of breast cancer. For RAD51-135C heterozygotes, HR = 3.46 (95% CI 1.3–9.2, $P = 0.01$). All *BRCA2* carriers who also were *RAD51-135C* heterozygotes became affected by age 58, whereas 50.4% of those who were *RAD51-135* normal homozygotes remained unaffected at the same age. (*C*) Fraction of *BRCA2* carriers remaining free of ovarian cancer. For *RAD51-135C* heterozygotes, HR = 1.23 (NS).

time breast cancer risk, a large study found a suggestion of lower risk in *BRCA2* carriers under 50 years of age (3). In Ashkenazi Jews, there is evidence that the *BRCA2–6174delT* mutation may be associated with lower cancer risk than the *BRCA1* mutations common in this population (*185delAG* and *5382insC*). *BRCA2–6174delT* is the most common mutation at the population level, with an estimated carrier frequency of 1.4%, compared with 1.1% for both *BRCA1* mutations combined (15). However, among affected carriers, the frequency of *BRCA2* mutations is approximately half that of *BRCA1* mutations, suggesting that many *BRCA2* carriers remain unaffected (ref. 15; Table 2). In this study, in which families were ascertained mostly on the basis of moderate family history (the mean number of affected relatives, excluding the proband, was 1.8), the *BRCA2–6174delT* mutation was approximately half as frequent as the *BRCA1–185delAG* and *5382insC* mutations combined.

The biological effect of the *RAD51-135C* polymorphism is yet to be elucidated and will be important to investigate. It is located in the 5′ untranslated region of the *RAD51* gene and theoretically could affect mRNA stability and/or translation efficiency, leading to altered RAD51 protein levels. Altered RAD51 levels could influence the activity of the multiprotein DNA-repair complex that includes BRCA1, BRCA2, and RAD51. Indeed, recent studies suggest altered *RAD51* expression may play a role in breast cancer pathogenesis. In breast carcinomas, loss of heterozygosity at the *RAD51* locus has been reported in 32% (41 of 127) (16), and reduced Rad51 protein levels have been found in 30% (54 of 179) (17). However, histological grading of sporadic invasive ductal carcinoma has been correlated with overexpression of wild-type Rad51 (18). In one case report, a missense mutation in *RAD51* (Arg-150–Glu) has been associated with bilateral breast cancer in two Japanese patients (12), although it is unknown whether this mutation segregated with the disease in their families and whether they were also carriers of *BRCA1* or *BRCA2* mutations. In our study, *RAD51-135C* was not associated specifically with bilaterality of breast cancer, although this result could be due to the small number of bilateral breast cancer cases. Among *BRCA2* carriers, the frequency of *RAD51-135C* heterozygotes in women with bilateral breast cancer was 1 in 5 (20%), similar to 17.9% (5 in 28) in unilateral cases (Table 1). Among *BRCA1* carriers, none of the 14 women with bilateral breast cancer were *RAD51-135C* heterozygotes, compared with 9.4% (6 of 64) of those with unilateral breast cancer.

The differential effect of *RAD135C* on *BRCA2* vs. *BRCA1* may be related to the different pathways in which the BRCA proteins play a role. Although both BRCA1 and BRCA2 are involved in DNA double-strand break repair by means of homologous recombination (a process requiring RAD51), modulation of RAD51 may not be as crucial for BRCA1 compared with BRCA2-mediated repair (reviewed in ref. 19). Conversely, as suggested by the observation that some *BRCA1* mutations are more severe than *BRCA2* mutations, DNA repair may be so impaired in the presence of BRCA1 defects that more minor alterations in RAD51 have no detectable impact. Our results also raise the possibility that *RAD51-135C* may have a differential effect on breast vs. ovarian cancer risk. The lack of effect on ovarian cancer could be the result of the small number of ovarian cancer outcomes in *BRCA2* carriers in this study. However, if it is confirmed in a larger series, it may be explained by differences in downstream targets in breast compared with ovarian tissues.

Current breast cancer prevention strategies in *BRCA2* carriers include chemoprevention with tamoxifen, prophylactic mastectomy, and probably prophylactic oophorectomy (20–22). Tamoxifen is expected to reduce breast cancer risk by up to 49%,

Table 2. Relative frequency of the common *BRCA1* and *BRCA2* mutations in Ashkenazi Jewish subjects and families affected with breast/ovarian cancer

Study subjects	Number	Affected *BRCA1* carriers, %	Affected *BRCA2* carriers, %	Ref.
Unselected living breast cancer cases	412	34 (8.3%)	15 (3.6%)	23
Unselected breast cancer cases, pathology slides	268	10 (3.7%)	8 (3%)	6
Unselected ovarian cancer cases	208	57 (27%)	29 (14%)	24
Selected breast cancer cases	178	16 (9%)	8 (4.5%)	25
Breast and breast/ovarian families	220	91 (41%)	9 (4%)	26
Families with known *BRCA1*/*BRCA2* mutations	205	141 (69%)	64 (31%)	This study
Breast or ovarian cancer survivors among Jewish volunteers	302	16 (5.3%)	11 (3.6%)	5

although its efficacy has not been demonstrated specifically in *BRCA1* or *BRCA2* carriers (20). Prophylactic mastectomy is effective in reducing breast cancer incidence by 90%, but this reduction is counterbalanced by the psychological impact of such surgery and the large number of mastectomies probably needed for each life saved (21). If the association of *RAD51-135C* with breast cancer risk in *BRCA2* carriers is confirmed in additional studies, *RAD51* status may be useful in differentiating those carriers most likely to benefit from aggressive prevention measures from those in whom more conservative management would be appropriate.

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