NIH Public Access
Author Manuscript
Fam Cancer. Author manuscript; available in PMC 2013 September 01.

Published in final edited form as:
Fam Cancer. 2012 September ; 11(3): 381–385. doi:10.1007/s10689-012-9523-9.

Mutation screening of *RAD51C* in high-risk breast and ovarian cancer families

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Abstract

Deleterious mutations in the *RAD51C* gene, which encodes a DNA double-strand break (DSB) repair protein, have been reported to confer high-penetrance susceptibility to both breast and ovarian cancer. To confirm this we conducted a mutation screen of the *RAD51C* gene in 192 probands from high-risk breast and/or ovarian cancer families that do not carry *BRCA1* or *BRCA2* mutations. The nine exons of the *RAD51C* gene containing protein coding sequence were screened for mutations in genomic DNA from family probands by high-resolution melting (HRM) analysis and direct DNA sequencing. Four missense variants, p.Ser364Gly, p.Ala126Thr, p.Val169Ala, and p.Thr287Ala were detected in six patients. The p.Ser364Gly variant is a novel variant predicted to have little influence on RAD51C activity. The p.Ala126Thr and p.Val169Ala variants have been reported to have no association with risk of breast cancer in a case-control study. However, p.Thr287Ala disrupts the DNA repair activity of RAD51C, suggesting some influence on risk. Consistent with published results from similar follow-up studies, we suggest that *RAD51C* mutations are rare events among high-risk breast cancer and breast/ovarian cancer families. Large population-based studies will be needed to reliably assess the prevalence and penetrance of inactivating mutations in the *RAD51C* susceptibility gene.

Keywords

mutation screening; *RAD51C*; familial breast and ovarian cancer; breast cancer predisposition

INTRODUCTION

Germline mutations in many of the genes that are involved in homologous recombination (HR)-mediated DNA double-strand break repair have been associated with predisposition to breast and ovarian cancer[1]. Together these genes, including the *BRCA1* and *BRCA2* susceptibility genes, confer up to 65% cumulative risk of breast cancer and 25% risk of ovarian cancer by age 80[2]. Rare inactivating mutations in several other genes in the DSB repair pathway, such as *ATM*[3], *BRIP1*[4], *PALB2*[5], *NBS1*[6], *RAD50*[7], and *TP53*[8] among others, have also been implicated in the development of breast cancer. These contribute to a small additional fraction of disease, leaving the genetic etiology of 50% of high-risk families unexplained. It is therefore likely that unidentified pathogenic mutations

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in other DNA damage and repair genes may contribute to the remaining susceptibility of breast and ovarian cancers in these families.

In mammalian cells, RAD51 acts in concert with BRCA1 and BRCA2 in HR to repair DSBs and ensure genomic stability. RAD51 interacts directly with BRCA2, which mediates RAD51 polymerization at sites of double strand breaks. In addition to RAD51, the RAD51B (RAD51L1), RAD51C, and RAD51D members of the RAD51 family are essential for HR activity. In particular, RAD51C has been implicated in DNA recombination repair as a component of two distinct complexes that bind single-stranded DNA, one in association with RAD51B, RAD51D, and XRCC2, and the other in association with XRCC3[9, 10]. Recently, six monoallelic truncating, splicing, and missense mutations in the *RAD51C* gene were identified in a single study of individuals from families with both breast and ovarian cancers. Four of the RAD51C missense variants only partially rescued mitomycin C (MMC) sensitivity of chicken DT40 cells that lacked endogenous *RAD51C*[11]. Separately, a biallelic missense mutation in *RAD51C* was identified in an individual exhibiting Fanconi-anemia like syndrome[12]. Since then, several large-scale follow-up studies further identified eight novel truncating mutations and a number of pathogenic missense variants in Spanish, Finnish, Swedish, and Australian familial breast and/or ovarian cancer patients with no *BRCA1* and *BRCA2* mutations[13–17]. In contrast, other studies of smaller numbers of male and female breast cancer cases from predominantly high-risk breast cancer families did not identify additional *RAD51C* mutations[18–22]. Taken together, data from these studies suggest that the overall mutation frequency of *RAD51C* gene in familial breast cancer is low. Here, we report results from a mutational analysis of the *RAD51C* gene in 192 high-risk breast and ovarian cancer families recruited at the Mayo Clinic between 1998 and 2006.

MATERIALS AND METHODS

Study Cohorts

This study was restricted to probands affected with breast cancer from high-risk families with at least two first- and second-degree relatives diagnosed with breast and/or ovarian cancers identified between 1998 and 2006 at the Mayo Clinic. A total of 46 probands were from high-risk breast and/or ovarian cancer families. *BRCA1* and *BRCA2* gene sequencing analysis by Myriad Genetics Laboratories, Inc. failed to identify germline pathogenic *BRCA1* or *BRCA2* mutations in DNA samples from these individuals. Another 146 probands were identified through a study of individuals carrying *BRCA1* and *BRCA2* variants of uncertain significance (VUS). All variants were assessed for pathogenicity using a sequence alignment-based Align-GVGD algorithm that incorporates information on the evolutionary sequence conservation of mutated residues and the physicochemical properties of the amino acid changes and yields a prior probability of causality [25]. Separately variants were assessed for pathogenicity using a posterior probability model [24] that incorporates the prior probability of causality and the likelihood of causality based on segregation with disease, co-occurrence with known pathogenic variants, and family history of breast and ovarian cancer. All probands were self-reported as Caucasian. Information collected included the year of birth, ages at breast and ovarian cancer diagnosis and family history of cancer. All patients provided signed informed consent. Both studies were approved by the Mayo Clinic Institutional Review Board (IRB).

DNA Specimen and Extraction

Blood samples obtained from 192 probands were processed by the Biospecimen Accessioning and Processing (BAP) Shared Resource at the Mayo Clinic Cancer Center. Genomic DNA was resuspended in 1X TE buffer at a uniform concentration of 10 ng/μl.

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PCR Amplifications, High-Resolution Melting Assays and DNA Sequencing

The forward and reverse primers for amplicons of the nine exons of *RAD51C* are shown in Supplementary Table 1. The length of most PCR products was less than 300 bp and the largest amplicon was 379 bp. The optimal temperature was determined for each pair of primers. PCR reactions were completed for each genomic DNA sample in 5 l volume with 1X amplification buffer (Qiagen, Valencia, CA), 0.3 mmol/L each deoxynucleoside triphosphate, 1 mmol/L $MgCl_2$, 0.1 unit/μL HotStar DNA polymerase (Qiagen), 0.5 μL LcGreen+ dye (Idaho Tech, Salt Lake City, UT), and 200 nmol/L forward and reverse primers. PCR conditions for each amplicon included 95°C for 15 minutes and 45 cycles at 95°C for 30 seconds, annealing temperature for 30 seconds, and amplification at 72°C for 30 seconds. Post-PCR plates were scanned directly in a LightScanner mutation analyzer (Idaho Tech) within a melt range of 70°C–95°C. Sample showing melt curve shifts resulting from the formation of mutant/wild-type heteroduplexes were considered positive. New PCR reactions without LcGreen+ dye were conducted for these positives and products were subjected to direct Sanger DNA sequencing to identify the exact nucleotide changes.

In silico analyses by SIFT, PolyPhen, and PMut

Amino acid substitutions were predicted to be neutral or pathogenic by web-based programs SIFT, PolyPhen, PMut, and Align-GVGD (SIFT: http://blocks.fhcrc.org/sift/SIFT.html. PMut: http://mmb2.pcb.ub.es:8080/pmut. Polyphen: http://genetics.bwh.harvard.edu/pph. Align-GVGD: http://agvgd.iarc.fr/agvgd_input.php). The prediction scores and the associated reliability indexes of these prediction programs compared favorably.

RESULTS

In this study we screened 192 probands of moderate to high-risk breast cancer families for *RAD51C* mutations. Table 1 outlines the phenotypic characteristics of the 192 families represented by the probands. Two probands were male breast cancer cases, 181 were female breast cancer cases, and nine were affected by both breast and ovarian cancers. A total of 157 probands had a family history of breast cancer only, whereas 35 had a family history of both breast and ovarian cancer. In addition, 166 of 192 families had three or more first- or second-degree relatives diagnosed with breast and/or ovarian cancers. The remaining 26 families had two incident breast cancer cases among first- and second degree relatives. On average, probands were diagnosed at age 46, which is similar to the published data from other cohorts of high-risk families of breast cancer, including those with *BRCA1* and *BRCA2* mutations.

Among these 192 families, 146 were recruited from a study of VUS in *BRCA1* and *BRCA2* genes. Each of the 146 probands used in the *RAD51C* mutation screen carried either a missense or a splicing variant in *BRCA1* or *BRCA2* with three probands carrying variants in both *BRCA1* and *BRCA2*. A summary of the predicted pathogenicity of the variants is shown in Table 2. A total of 49 missense substitutions and two intronic changes were shown by genetic and functional studies to be non-pathogenic Class 1 or Class 2 alterations (Table 2) [23–25]. A further 61 missense variants remained unclassified (Class 3), with prior probabilities of causality ranging from 0.02 to 0.81. Of these, 55 missense alterations were graded as C0 or C15 variants by the Align-GVGD algorithm with low prior probabilities of pathogenicity (<0.29), whereas six were graded as C46-C65 (prior probability 0.66–0.81). Eight intronic alterations not located in splice donor or acceptor sites were also considered unclassified (Class 3). One variant (BRCA1 R1495M) was classfied as pathogenic (Class 5) after the completion of mutation screening. These data suggest that the great majority of these 146 probands do not carry pathogenic *BRCA1* or *BRCA2* mutations.

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PCR conditions for amplification of the nine *RAD51C* exons are listed in Supplementary Table 1. The amplicons covered all 5′UTR, 3′UTR and coding sequences and exon-intron boundaries of *RAD51C* full-length cDNA (NM_058216.1). No insertions, deletions or splicing mutations were identified in the 192 DNA samples in the high-resolution melting (HRM) scan. However, four rare missense mutations were identified in exons 2, 3, 6, and 9 of *RAD51C* in five of the probands from the VUS study probands and a single proband from a high-risk family with no VUS (Table 3). The six probands were from high-risk families with two to five first- and second-degree relatives diagnosed with breast cancer only. The p.Ala126Thr, p.Val169Ala and p.Thr287Ala were previously reported to have no association with breast cancer risk in a breast cancer case-control study[11]. The novel variant, p.Ser364Gly in exon 9, was evaluated by PMut, SIFT and PolyPhen and was predicted to have no effect on the function of the RAD51C protein.

DISCUSSION

We did not identify any clearly inactivating protein truncating mutations in a screen of all the exonic sequences and exon-intron boundary sequences of the *RAD51C* gene. Two missense variants, p.Ala126Thr and p.Val169Ala, detected in the mutation screen were previously reported to have no influence on the contribution of RAD51C to DNA repair (Table 3). The novel variant p.Ser364Gly has not been observed in any previous mutation screening studies of *RAD51C*, but is most likely a neutral variant based on *in silico* prediction algorithms. In contrast, the p.Thr287Ala variant was predicted by PolyPhen to have a damaging effect on the function of RAD51C protein, and was shown to have a reduced ability to complement MMC sensitivity in *Rad51c* null chicken DT40 cells[11]. However, this variant was reported to have no association with breast cancer risk in a breast cancer case-control study [11]. The functional effect contrasts with the results of the case-control study and suggests that inactivating mutations in this gene may not influence risk in the same manner as inactivating BRCA1 or BRCA2 mutations. Instead, RAD51C mutations may be associated with much lower risk of breast or ovarian cancer similarly to CHEK2 and ATM. Much larger, appropriately matched, case-control studies will be needed to determine the influence of mutations like T287A on cancer risk.

The absence of *RAD51C* mutations from our mutation screen and null results reported in several other follow-up studies support the initial data showing a lack of mutations in high-risk breast cancer families. Instead inactivating *RAD51C* mutations have been observed almost exclusively in high-risk families diagnosed with both breast and ovarian cancers [11, 13, 15, 16]. More recently it has been suggested that *RAD51C* mutations are associated predominantly with a risk of ovarian cancer [14]. Since our cohort had a limited number of families with both breast and ovarian cancer cases (n=35), of which 19 were from the VUS cohort, this is a plausible explanation for the absence of inactivating *RAD51C* mutations from our screening study.

Interestingly, this enhanced susceptibility to ovarian cancer is further supported by the recent discovery of truncating mutations in the *RAD51D* gene that were found to display strong associations with risk of ovarian cancer in a cohort of 911 high-risk breast and/or ovarian cancer families [26]. Since RAD51C and RAD51D exist in a complex containing RAD51L1 and XRCC2 that mediates HR repair of DNA double strand breaks, it appears that disruption of this complex may have a greater influence on ovarian cancer than breast cancer. Whether this points to more limited secondary repair systems in ovarian than breast epithelial cells, or an overall greater sensitivity to accumulating DNA damage in ovarian epithelial cells remains to be determined.

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Further consideration of the contribution of *RAD51C* to familial breast and ovarian cancer is merited with more focus on ascertaining ovarian cancer families. The absence of *RAD51C* gene mutations from our mutation screen suggests that deleterious mutations in other DNA damage repair genes, such as *BRIP1*, *PALB2*, *NBS1*, and *RAD50*, among others, may account for the increased susceptibility in our cohort of high-risk *BRCA1/2* mutation negative families. Furthermore, mutations in additional, as yet undefined, susceptibility genes are likely to exist in these families. Future efforts should be directed at identification of these susceptibility genes to obtain a full spectrum of knowledge of genetic etiology of hereditary breast and ovarian cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Breast Cancer Research Foundation

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Table 1

Characteristics of 192 high-risk breast cancer families

Age at first cancer diagnosis of proband (years; mean ± SD)	46.2 ± 9.2
First- and second-degree relatives with breast cancer per family (mean ± SD)	3.6 ± 1.5
First- and second-degree relatives with ovarian cancer per family (mean ± SD)	1.4 ± 0.8
First- and second-degree relatives with breast and/or ovarian cancers per family (mean ± SD)	3.8 ± 1.6
Families with 6 first- and second-degree relatives with breast and/or ovarian cancers (n)	24
Families with 3–5 first- and second-degree relatives with breast and/or ovarian cancers (n)	142
Families with 2 first- and second-degree relatives with breast and/or ovarian cancers (n)	26
Families with history of breast cancer only (n)	157
Families with history of breast and ovarian cancers (n)	35
Probands diagnosed with both breast and ovarian cancers (n)	9
Gender of probands (female/male) (n)	190/2

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Table 2

Classification of BRCA1/2 variants identified in 146 high-risk families

Gene	Non-pathogenic Class 1 or 2	Pathogenic Class 4 or 5	Unclassified Class 3 VUS	Intronic variants		
				Class 1 or 2	Class 4 or 5	Unclassified
BRCA1	21	1	18	0	0	3
BRCA2	28	0	43	2	0	5

Note: three probands have both BRCA1 and BRCA2 VUS.

Fam Cancer. Author manuscript; available in PMC 2013 September 01.

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Table 3

Nucleotide and amino acid substitutions identified in probands from 192 high-risk breast cancer families

Site	* Nucleotide change	* Amino acid change	Number of probands (n)	Predictive algorithms		
				SIFT	PolyPhen	Pmut
Exon 2	c. 376G>A	p.Ala126Thr	2#	Tolerated	Benign	Neutral
Exon 3	c.506T>C	p.Val169Ala	1#	Tolerated	Benign	Neutral
Exon 6	c.859A>G	p.Thr287Ala	2#,$	Tolerated	Damaging	Neutral
Exon 9	c.1090A>G	p.Ser364Gly	1#	Tolerated	Benign	Neutral

*HGVS nomenclature;

#probands from cohort of 146 families;

$proband from cohort of 46 families.

Fam Cancer. Author manuscript; available in PMC 2013 September 01.