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Loss of function germline mutations in *RAD51D* in women with ovarian carcinoma

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Abstract

Objective—*RAD51D*, a gene in the Fanconi Anemia-BRCA homologous recombination pathway, has recently been shown to harbor germline mutations responsible for ovarian carcinoma in multiply affected families. We aimed to extend these results to ovarian carcinoma in the general population.

Methods—We sequenced *RAD51D* in germline DNA from 360 individuals with primary ovarian, peritoneal or fallopian tube carcinoma who were not selected for age of cancer onset or family history. We also sequenced *RAD51D* in 459 probands from 226 high risk breast cancer families who were wild type for 21 breast and ovarian cancer genes.

Results—Of 360 cases, three (0.8%) carried loss-of-function mutations in *RAD51D*. All three subjects had ovarian carcinoma; one was also diagnosed with a synchronous endometrial carcinoma. Only one of the three subjects had a family history of breast or ovarian cancer. Combined with previous data for this series, 23.9% of women with unselected ovarian, fallopian tube, or peritoneal carcinoma carried a germline loss-of-function mutation in any of 13 tumor suppressor genes. Among the 449 women and 10 men with familial breast cancer, none carried a loss of function mutation in *RAD51D*.

Conclusions—These data support the previous observation that loss-of-function mutations in *RAD51D* predispose to ovarian carcinoma but not to breast carcinoma. We conclude that inherited ovarian cancer is highly heterogeneous genetically, and that approximately one in four ovarian

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Conflicts of interest statement

The authors have no conflicts of interest to declare.

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carcinoma patients carry a germline mutation in a known tumor suppressor gene that confers high risk.

Introduction

Ovarian carcinoma has the highest mortality of all gynecological cancers. Nearly one in four ovarian, fallopian tube, and peritoneal carcinomas are associated with germline loss-of-function mutations in tumor suppressor genes: approximately 18% *BRCA1* or *BRCA2*; 5% in other genes of the Fanconi Anemia-BRCA DNA repair pathway, including *MRE11A*, *BRIP1*, *PALB2*, *RAD51C*, *CHEK2*, *NBN*, *RAD50*, and *BARD1*; and less than 1% in genes of DNA mismatch repair pathways [1]. Identification of mutations predisposing to high risk of ovarian carcinoma enables women carrying such alleles to consider appropriately timed salpingo-oophorectomy, which significantly reduces mortality rates among genetically high-risk women [2].

Recently, *RAD51D* was added to the set of genes predisposing to ovarian carcinoma [3]. *RAD51D* is one of five paralogs of *RAD51*, a DNA recombinase involved in homologous recombination (HR) in eukaryotic cells [4]. *RAD51D*-deficient cells are sensitive to poly-ADP-ribose polymerase (PARP) inhibitors, similar to *BRCA1*-deficient and *BRCA2*-deficient cells, and consistent with an HR-deficient phenotype [3]. Germline loss-of-function mutations in *RAD51D* were identified in eight of 911 probands from families with both breast and ovarian cancer, in zero of 737 probands from families with breast cancer only, and in one of 1060 control subjects. The probands from these high-risk families had all previously tested negative for *BRCA1* and *BRCA2* mutations. The relative risk of ovarian cancer for *RAD51D* mutation carriers was estimated at 6.30 (95% CI 2.86–13.85), a clinically meaningful level of risk. In a second, confirmatory study of families with both breast and ovarian cancer, loss-of-function *RAD51D* mutations were identified in one of 175 *BRCA1/2* negative families with both breast and ovarian cancer [5].

To extend these results, we undertook the present project to estimate the frequency of *RAD51D* mutations among subjects with ovarian, fallopian tube, or peritoneal carcinoma not selected for family history of the disease. In addition we sequenced *RAD51D* in an independent series of breast cancer cases selected for family history and wild type for the 21 tumor suppressor gene panel previously tested in our ovarian series.

Methods

All participants provide informed consent for genetic analysis as approved by the human subjects committee of the University of Washington Institutional Review Board. Two groups of subjects were evaluated. One group comprised 360 women, not selected for age of onset of cancer or family history of cancer, who underwent surgery for primary ovarian, fallopian tube or peritoneal carcinoma at the University of Washington as previously described [1]. The second group comprised 449 women and 10 men with primary invasive breast carcinoma from 226 families with at least four breast and/or ovarian cancers [7]. All subjects were previously screened for germline mutations in 21 breast or ovarian carcinoma tumor suppressor genes using our BROCA test that includes *BRCA1*, *BRCA2*, *BRIP1*, *BARD1*, *CHEK2*, *MRE11A*, *NBN*, *RAD50*, *RAD51C*, *PALB2*, *TP53*, *PTEN*, *STK11*, *CDH1*, *ATM*, *MLH1*, *MSH2*, *MSH6*, *PMS1*, *PMS2*, and *MUTYH*. Results of this testing for ovarian cancer cases has been published [1]. All 459 breast cancer patients were negative for deleterious mutations in the 21 tumor suppressor genes using BROCA. All participants provided informed consent for genetic studies (University of Washington protocol 34173). DNA was extracted from blood or lymphoblast cell lines as previously described [1, 7]. *RAD51D* was analyzed by Sanger sequencing using PCR primers to amplify all exons and

intron-exon boundaries of the gene (Supplementary Table 1). PCR amplicons were sequenced bidirectionally using Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI 3130xl Genetic Analyzer. Trace sequences were analyzed using Sequencher 4.9 software. Mutations were confirmed in a second separate PCR and sequencing reaction. DNA from tumors of subjects with *RAD51D* mutations was sequenced to assess loss of heterozygosity (LOH). LOH was determined by comparing the ratio of peak heights in normal versus neoplastic DNA. To analyze splice-site mutations, total RNA was isolated from patients' whole blood using Invitrogen TRIzol Plus RNA Purification System and complementary DNA (cDNA) was generated by gene-specific priming using Invitrogen Superscript II reverse transcriptase. The cDNA was amplified with nested PCR using two sets of primer pairs spanning exons 1–4 and exons 1–3. The products were electrophoresed on a 2% agarose gel and the resolved DNA fragments extracted from the gel using a Qiagen QIAquick Gel Extraction Kit and sequenced bidirectionally using Applied Biosystems BigDye Terminator v3.1.

Results

Of the 360 subjects with primary ovarian, peritoneal, or fallopian tube carcinoma, three (0.83%) carried loss of function mutations in *RAD51D* (Table 1). Mutations were one nonsense, one frameshift, and one splice-site substitution leading to loss of exon 2 and a frameshift in the *RAD51D* transcript (Figure 1). None of the mutations have been reported previously. The three subjects with *RAD51D* mutations were aged 33, 43 and 75 years old at diagnosis. One subject had a mother and maternal grandmother with post-menopausal breast cancer and a paternal aunt with early onset ovarian cancer; the other two subjects had no family history of breast or ovarian cancer. All three subjects with *RAD51D* mutations had Grade 3 ovarian carcinomas; one subject was diagnosed with both ovarian carcinoma and a separate synchronous endometrial carcinoma (Table 1). This subject was one of only eight patients with this clinical presentation. One of the three ovarian carcinomas from these subjects had loss of the wildtype allele at the *RAD51D* mutation site (Figure 1). All three subjects had wildtype sequences for the 21 other known ovarian and breast cancer tumor suppressor genes [1].

Combined with previous data from this series of subjects with ovarian, fallopian tube or peritoneal cancer, there are 89 loss-of-function germline mutations identified in known tumor suppressor genes in 86 of the 360 individuals. This increased the percentage of individuals with a damaging mutation from 23.1% to 23.9%.

In addition to the three subjects with loss-of-function mutations in *RAD51D*, five subjects carried rare missense variants in *RAD51D*: p.C9S, p.S46C, p.S207L, p.R239W, p.I311N (Table 2). Three of the missense alleles (p.S46C, p.S207L, p.I311N) were predicted to be damaging by both PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>) and SIFT (<http://sift.jcvi.org/>) (Table 2). Single nucleotide polymorphisms (SNPs) in the *RAD51D* coding sequence were observed at frequencies similar to those reported: rs9901455 (allele frequency 0.097), rs4796033 (0.140), rs28363284 (0.014), and rs28363283 (0.001).

In contrast, none of the 459 familial breast cancer patients carried loss of function mutations or rare missense variants in *RAD51D*. Among the familial breast cancer patients, three of the four SNPs were observed: rs9901455 (allele frequency 0.095), rs4796033 (0.139), and rs28363284 (0.011).

Discussion

We identified germline *RAD51D* mutations in 1% of subjects with ovarian carcinomas not selected for age of diagnosis or family history, but in zero of 449 subjects with familial breast cancer, consistent with the previous identification of *RAD51D* as an ovarian but not breast cancer gene [3, 5]. These results extend the role of *RAD51D* to the general population of subjects with ovarian carcinoma. Two previous studies demonstrated an increased frequency of *RAD51D* mutations in families with two or more cases of ovarian cancer [3, 5]. Analysis only of familial ovarian cancer would have missed two of the three mutation carriers in the present series. Adding the *RAD51D* mutations to previously identified loss of function mutations in this series brings the total number of genes harboring germline mutations in ovarian cancer patients to 13 and the total proportion of patients with a deleterious mutation to 23.9%, including *BRCA1* or *BRCA2* as well as other tumor suppressor genes (*BARD1*, *BRIP1*, *CHEK2*, *MRE11A*, *MSH6*, *NBN*, *PALB2*, *RAD50*, *RAD51C*, *RAD51D*, and *TP53*). Including the other known Lynch syndrome genes (*MSL1*, *MSH2* and *PMS2*), at least 16 genes contribute to ovarian cancer risk. These data support offering comprehensive genetic evaluation for all known ovarian tumor suppressor genes to all women with ovarian carcinoma, regardless of age at onset or family history.

The role of rare missense variants in *RAD51D* in ovarian cancer risk is not yet clear. Rare *RAD51D* missense variants appear also in controls, and one of the missense variants in this series occurred in a subject who also carried a deleterious mutation (Table 2). One of five neoplasms with a missense variant (p.C9S) revealed loss of the wildtype allele, but this variant occurred at similar frequency in European American controls and was predicted by SIFT to be benign. Putting our data together with previous reports, *RAD51D* mutations have occurred with LOH of the wildtype allele in four of seven ovarian carcinomas [3, 5]. We conclude that somatic genomic deletion of the wildtype *RAD51D* allele is not required in ovarian carcinomas associated with germline loss-of-function *RAD51D* mutations and should not be used to determine the nature of missense variants. A functional assay for *RAD51D* missense variants, and data on co-segregation of missense variants with ovarian cancer in families could help clarify the impact of these variants.

The ages at onset of ovarian cancer among subjects with *RAD51D* mutations, both from our series (Figure 2) and those previously [3], suggest that preventing *RAD51D*-associated ovarian cancer will require offering risk-reducing salpingo-oophorectomy to unaffected mutation carriers at a relatively early age. It is difficult to make definitive recommendations for timing of preventive surgery with so few *RAD51D* mutation carriers identified to date. At present, we suggest recommendations similar to those for women with *BRCA1* mutations: salpingo-oophorectomy at age 35–40 after completion of childbearing unless earlier onset of ovarian carcinoma is documented in the family. The presence of a *RAD51D* mutation in one of eight women with synchronous endometrioid ovarian and endometrial cancers suggests that *RAD51D* should be evaluated in a larger series of women with this clinical presentation. Until more data is available, we would recommend concurrent hysterectomy with risk-reducing salpingo-oophorectomy.

All three subjects with *RAD51D* mutations had a complete response to platinum based chemotherapy and are currently without evidence of disease from 16 to 109 months post diagnosis. Interestingly, the individual who is currently without disease at 109 months presented initially with stage IV disease with parenchymal liver and splenic metastases. She had a complete response to cytoreductive surgery and platinum based therapy, but at 43 months, she had a large volume recurrence in the small bowel and was treated with secondary surgical cytoreduction and combination chemotherapy. She remains without further evidence of any recurrence five years later. Presumably, cancer cells with *RAD51D*

mutations would have defective homologous recombination mediated DNA repair, as evidenced by the demonstration of *in vitro* sensitivity to PARP inhibitors in RAD51D deficient cells [3]. The excellent therapeutic response of our *RAD51D* mutated cases to platinum based chemotherapy is consistent with a defect in DNA repair by homologous recombination.

RAD51D mutations account for a small yet clinically actionable proportion of hereditary ovarian carcinoma. The relative risk of ovarian cancer of approximately 6-fold for women with *RAD51D* mutations is clinically important and should be addressed with targeted prevention in at-risk individuals. Comprehensive assessment of ovarian cancer risk should include evaluation of *RAD51D* in a multigene panel. We have added *RAD51D* to the latest version of our multigene panel, BROCA [1, 6].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *RAD51D* loss-of-function mutations are present in 1% of unselected women with ovarian cancer.
- Ovarian cancer risk assessment should include evaluation of *RAD51D* in a multigene panel.
- Women with ovarian cancer should be evaluated for hereditary risk without selection for family history.

Figure 1. Trace Sequences

DNA sequence traces of paired germline and neoplastic DNA from cases with deleterious *RAD51D* mutations. Retention of heterozygosity at the mutation site is evident in the carcinomas from CF684.01 and 820.01, but the carcinoma from CF 1898.01 has loss of the wildtype allele. The sequence of the mutant transcript (cDNA) sequence for the splice-site mutation revealed skipping of exon 2 leading to a premature stop.

Figure 2. Age Distribution of Germline Mutations in Ovarian Cancer Genes

Proportion of subjects with ovarian, fallopian tube, or peritoneal carcinoma with a loss-of-function germline mutation in *RAD51D*, *BRCA1*, *BRCA2*, *BARD1*, *BRIP1*, *CHEK2*, *MRE11A*, *MSH6*, *NBN*, *PALB2*, *RAD50*, *RAD51C*, or *TP53* (see also Walsh et al. 2011).

Truncating Mutations in *RAD51D* in Ovarian Cancer Patients

Table 1

CF	Mutation (hg19) Start Position	Effect (cDNA, protein)	Age dx	Origin	Stage	Histology	Grade	Personal History Other Cancer	Family History	LOH	Survival (ongoing)
684.01	chr17:33,430,560	c.580delA, p.T194L_fsX6	34	ovary and endometrium	1C	endometrioid	3	synchronous endometrial carcinoma	no	no	32 months
820.01	chr17:33,430,296	c.694C>T, p.R232X	43	ovary	4	carcinoma	3	no	PA ov 60, M br 67, MGM br 50s	no	109 months
1898.01	chr17:33,446,115	c.131_144+24del38^, p.V28G_fsX21	75	ovary	3C	serous	3	no	S lung 65 (smoker)	yes	16 months

PA: Paternal Aunt; M: Mother; MGM: Maternal Grandmother; S: sister, ov, ovarian cancer; br, breast cancer; lung, lung cancer; LOH, Loss of Heterozygosity

^ splice-site mutation which leads to deletion of exon 2 and premature stop at codon 49.

Rare Missense Mutations in *RAD51D* in Ovarian Cancer Patients

Table 2

CF	Variant (hg19) Start Position	Effect (cDNA, Protein)	Age dx	Origin	Stage	Histology	Grade	LOH	Frequency among European Americans N=3510 ^{**}	Frequency among African Americans N=1870 ^{**}	Frequency among BC/OC cases N=1822 [^]	Frequency among controls N=2120 [^]	Polyphen-2 Score	SIFT Score
539.01	chr17:33,446,607	c.26G>C, p.C9S	72	ovary	3C	serous	3	loss WT	5	0	2	1	0.948	P= 0.40
1448.01	chr17:33,446,137	c.137C>G, p.S46C	61	ovary	3B	serous	3	No	2	0	1	0	1.000	P= 0.01
610.01	chr17:33,430,520	c.620C>T, p.S207L	58	peritoneal	3C	serous	3	No	1	0	1	0	1.000	P= 0.00
820.01 [*]	chr17:33,430,317	c.715C>T, p.R239W	43	ovary	4	carcinoma	3	No	0	0	0	0	0.999	P= 0.41
795.01	chr17:33,428,027	c.932T>A, p.I311N	66	peritoneal	3C	serous	3	No	2	0	0	0	0.996	P= 0.00

LOH, Loss of Heterozygosity; WT, Wildtype; EVS, Exome Variant Server (<http://evs.gs.washington.edu/EVS/>)

^{*} had a deleterious RAD51D mutation (Table 1)

^{**} frequencies from the Exome Variant Server (<http://evs.gs.washington.edu/EVS/>), N, number of persons sequenced (2N chromosomes)

[^] frequencies from reference 1