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RAD51C is a susceptibility gene for ovarian cancer

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A homozygous mutation in the *RAD51C* gene was recently found to cause Fanconi anemia-like disorder. Furthermore, six heterozygous deleterious *RAD51C* mutations were detected in German breast and ovarian cancer families. We screened 277 Finnish familial breast or ovarian cancer patients for *RAD51C* and identified two recurrent deleterious mutations (c.93delG and c.837+1G>A). These mutations were further genotyped in 491 familial breast cancer patients, 409 unselected ovarian cancer patients and two series of unselected breast cancer cases (884 from Helsinki and 686 from Tampere) and population controls (1279 and 807, respectively). The mutation frequency among all breast cancer cases was not different from the controls (4 out of 2239, 0.2% versus population controls 2 out of 2086, 0.1%, $P = 0.7$). In the Helsinki series, each mutation was found in four cases with personal or family history of ovarian cancer. No mutations were found among cases with familial breast cancer only, four out of the eight carriers did not have family history of breast cancer. The mutations associated with an increased risk of familial breast and ovarian cancer (OR: 13.59, 95% CI 1.89–97.6, $P = 0.026$ compared with controls), but especially with familial ovarian cancer in the absence of breast cancer (OR: 213, 95% CI 25.6–1769, $P = 0.0002$) and also with unselected ovarian cancer (OR: 6.31, 95% CI 1.15–34.6, $P = 0.033$), with a significantly higher mutation rate among the familial cases (two out of eight, 25%) than the unselected ovarian cancer cases (4 out of 409, 1%) (OR: 33.8, 95% CI 5.15–221, $P = 0.005$). These results suggest *RAD51C* as the first moderate-to-high risk susceptibility gene for ovarian cancer.

INTRODUCTION

Breast cancer is the most common cancer and the leading cause of cancer-related death among women worldwide, whereas ovarian cancer is the sixth most common cancer and the seventh cancer-related cause of death among women (1). Breast cancer is approximately twice as common among the first-degree female relatives of breast cancer patients as in the general population (2), making family history of breast cancer one of the strongest risk factors for the disease. Hereditary susceptibility to breast cancer is considered to be polygenic (3,4). The most important breast cancer susceptibility genes are *BRCA1* and *BRCA2* accounting for ~15% of familial predisposition to breast cancer (5). Mutations in these genes confer a high risk of early-onset breast cancer and also an increased risk of ovarian cancer (6,7). Increased breast or ovarian cancer risk is also present in other, rare cancer predisposition syndromes (8–11). Mutations in other breast cancer genes, *ATM*, *CHEK2*, *PALB2* and *BRIP1*, confer a more modest risk of breast cancer (12–15). Most of the familial ovarian cancer is also caused by germline mutations in the *BRCA1* and *BRCA2*

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Table 1. Frequencies of the detected *RAD51C* variations in the screening of 277 cancer patients

rs number	Variation	AA, (%)	Aa, (%)	aa, (%)	MAF	CEU
rs12946397	c.-26C>T	175 (63.2)	82 (29.6)	20 (7.22)	0.220	0.200
	c.93delG	275 (99.3)	2 (0.72)	0 (0)	0.0036	
	c.790G>A	276 (99.6)	1 (0.36)	0 (0)	0.0018	
	c.837+1G>A	275 (99.3)	2 (0.72)	0 (0)	0.0036	
rs28363317	c.859A>G	276 (99.6)	1 (0.36)	0 (0)	0.0018	0.017
rs28363318	c.904+34T>C	135 (48.7)	115 (41.52)	27 (9.75)	0.305	0.292

AA, frequency of the common homozygote; Aa, frequency of the heterozygote; aa, frequency of the rare homozygote; MAF, minor allele frequency in this study; CEU, minor allele frequency in the 1000 Genomes CEU population.

genes. However, no other high or moderate penetrance susceptibility genes for ovarian cancer as a main cancer phenotype have been identified so far (16). Several low-penetrance susceptibility loci for both breast (17–21) and ovarian cancer (22–24) have been identified through genome-wide association studies.

Biallelic mutations in some of the breast cancer susceptibility genes cause Fanconi anemia (FA), which is a rare chromosomal instability disorder characterized by developmental abnormalities, bone-marrow failure and susceptibility to leukemia and other cancers (25). To date, mutations have been identified in 13 genes in the known Fanconi anemia complementation groups. All of these genes have a role in DNA damage repair. Biallelic mutations in the *BRCA2*, *BRIP1* and *PALB2* genes cause Fanconi anemia subtypes D1, J and N, respectively (26–30). Most FA proteins are components of the FA core complex which is a large multiunit ubiquitin ligase that is required for monoubiquitination of FANCD2 and FANCI in response to DNA damage (25). The ubiquitin-tagged FANCD2–FANCI complex interacts with downstream FA proteins that are important for DNA damage repair through homologous recombination. Also BRCA1 works together with the FA proteins in the DNA-repair pathway (25,31).

Recently, a homozygous missense mutation in the *RAD51C* gene was found to cause Fanconi anemia-like disorder in a consanguineous family (32), while rare heterozygous mutations were found to confer an increased risk of breast and ovarian cancer with high penetrance (33). Meindl *et al.* (33) screened 1100 index cases from German families and found six pathogenic *RAD51C* mutations in families with breast and ovarian cancer but not in families with breast cancer only or in healthy controls.

RAD51C [*RAD51* homolog C (*S. cerevisiae*)], located on chromosome 17q23, is a member of the *RAD51* gene family, which encodes strand-transfer proteins (34). The *RAD51C* transcript (*RAD51C-001*, nomenclature according to Ensembl) is expressed in various human tissues, with the highest expression level in testis, followed by heart muscle, spleen and prostate. RAD51C is involved in homologous recombination (35) and, like BRCA2, PALB2 and BRIP1, it is thought to function downstream of FANCD2 and FANCI (32). RAD51C is also required for the activation of the checkpoint kinase CHEK2 (36).

To investigate the role of *RAD51C* mutations in Finnish breast and ovarian cancer families, we have here screened 277 patients from breast and/or ovarian cancer families for mutations in the *RAD51C* gene. Furthermore, we screened the identified mutations in additional breast and ovarian cancer families, and in breast cancer and ovarian cancer patient series unselected for family history, and performed haplotype analysis of the carriers and LOH analysis of the tumors of the mutation carriers.

RESULTS

Identification of the *RAD51C* mutations

We screened 277 familial *BRCA1/2* negative breast and ovarian cancer patients for germline variation in the *RAD51C* gene using bidirectional sequencing. One hundred and thirty of these patients belong to families with multiple breast cancer cases, 139 in families with both breast and ovarian cancer and eight in ovarian cancer families. Two heterozygous recurrent novel pathogenic germline mutations c.93delG and c.837+1G>A were identified in four families. One previously detected missense variant c.790G>A was found from a breast cancer patient with one first-degree relative affected with ovarian cancer. Also three known polymorphisms rs12946397, rs28363317 and rs28363318 were detected among the patients. The frequencies of the mutations and the polymorphisms are shown in Table 1.

The frameshift mutation c.93delG in *RAD51C* exon 1 was found in a breast cancer index case of a family with four breast cancer cases and one ovarian cancer case (Fig. 1D, pedigree 5) and in an ovarian cancer family with two ovarian cancer cases and no history of breast cancer (Fig. 1C, pedigree 6). The donor splice site mutation c.837+1G>A at the exon 5–intron 5 boundary was found in a breast cancer index case of a family with four breast and one ovarian cancer cases (Fig. 1A, pedigree 1) as well as in an ovarian cancer patient whose mother was also affected with ovarian cancer (Fig. 1B, pedigree 2).

Additional screening for the identified mutations

The identified mutations c.93delG and c.837+1G>A and the variant c.790G>A were further screened, using TaqMan assays or by sequencing the *RAD51C* exons 1 and 5, in an unselected series of 884 breast cancer patients, in 491 additional familial breast cancer patients, 409 unselected ovarian cancer patients and 1279 healthy female population controls from the same geographic region. Out of the unselected series of breast cancer patients, 833 patients who had

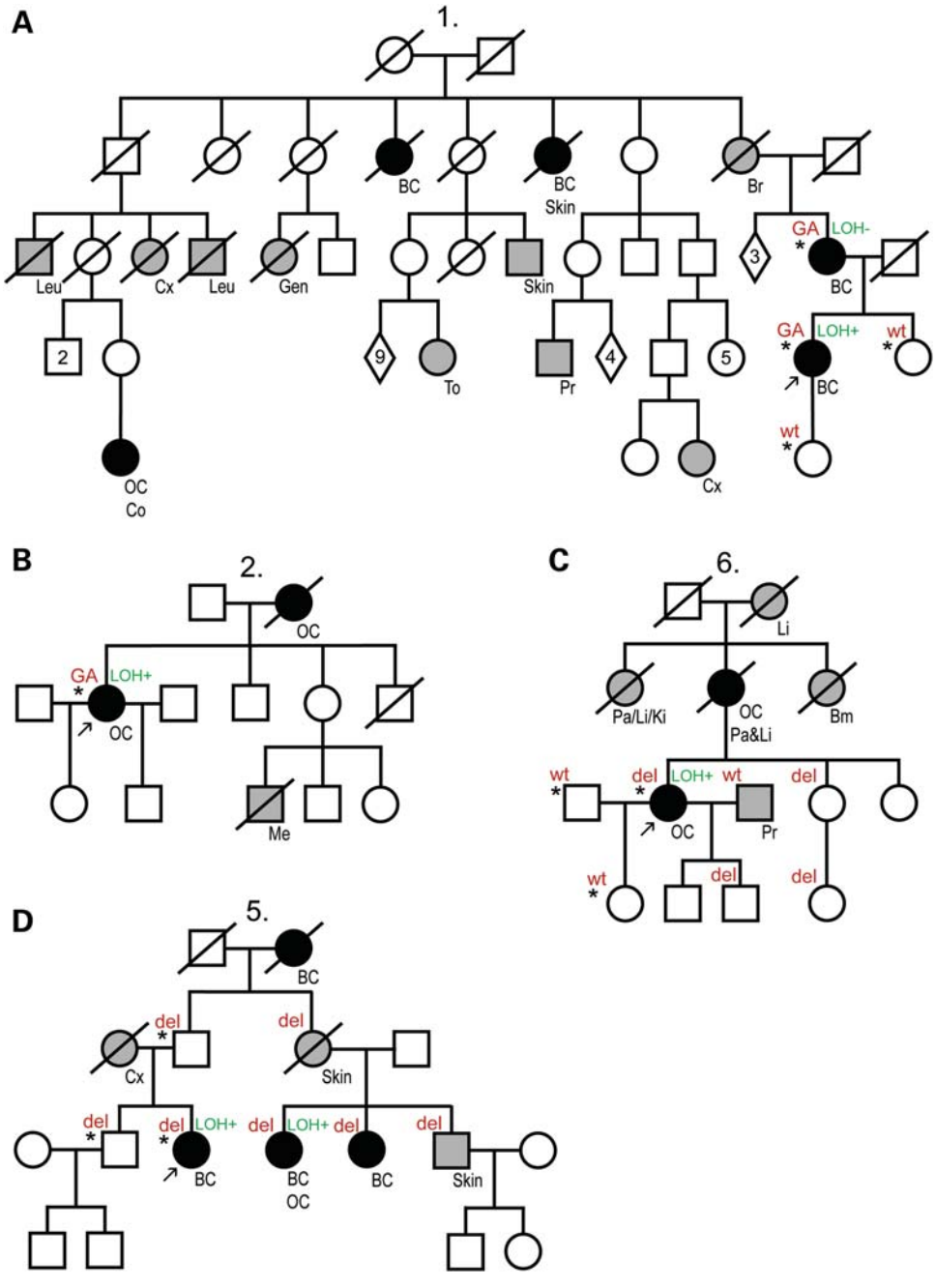

Figure 1. Pedigrees of *RAD51C* mutation carrier families. Subjects that were included in the haplotype analysis are denoted with an asterisk (*). BC, breast cancer; Bm, chronic bone marrow cancer; Br, brain cancer; Co, colon cancer; Cx, cervical cancer; Gen, female genital-tract cancer; Ki, kidney cancer; Leu, leukemia; Li, liver cancer; Me, melanoma; OC, ovarian cancer; Pa, pancreatic cancer; Pr, prostate cancer; Skin, skin cancer; To, tongue cancer; GA (red), c. 837+1G>A; del (red), c.93delG; wt (red), wild-type; LOH+ (green), loss-of-heterozygosity; LOH− (green), no loss-of-heterozygosity.

invasive breast cancer and reliable genotyping result were included in the analysis (98% of the invasive cases). The mutations c.93delG and c.837+1G>A were also further screened in an additional series of 686 unselected breast cancer patients and 807 healthy population controls from Tampere region of Finland.

The c.93delG mutation was further found in two unselected ovarian cancer patients. One of the mutation positive patients had a mother affected with ovarian cancer but did not have family history of breast cancer. The other mutation positive patient had no known family history of ovarian cancer, but she had a cousin affected with breast cancer. The c.837+1G>A mutation was also further found in two unselected ovarian cancer patients. Neither one of these patients had known family history of ovarian cancer, but one of them had a second-degree relative affected with breast cancer. The extended family history of cancer in all eight mutation carrier families is presented in Table 2.

Table 2. Family background of cancer in *RAD51C* mutation carrier families

Family	Mutation	Family history of BC	Family history of OC	Family history of other cancers
1	c.837+1G>A	4 (60y; 76y; **62y; 45y**)	1 (34y)	Colon (34y), 2 leukemia (67y; 84y), 2 cervical (48y; 27y), female genital (62y), skin of trunk (65y), skin (82y), tongue (51y), prostate (57y), brain (91y)
2	c.837+1G>A		2 (**53y**)	Melanoma (40y)
3	c.837+1G>A	1 (78; maternal side)	1 (**68y**)	Kidney (65y), stomach (50y; paternal side), undefined cancer from paternal side
4	c.837+1G>A		1 (**50y**)	
5	c.93delG	4 (74y; **55y; 76y; 63y**)	1 (**59y**)	Skin of upper limb and shoulder (**85y**), skin of trunk (**64y**)
6	c.93delG		2 (56y; **60y**)	Pancreas and liver (73y), pancreas/liver/kidney, chronic bone marrow, liver, brain
7	c.93delG		2 (**54y**; 64y)	Abdomen (paternal side), ventricle (maternal side), lung (maternal side), undefined cancer from maternal side
8	c.93delG	1	1 (**60y**)	Cervical (33y)

Age at diagnosis is denoted in brackets and mutation carriers are in bold-face.
BC, breast cancer; OC, ovarian cancer; y, years.

No additional mutation carriers were found among the unselected or familial breast cancer patients from Helsinki region. c.837+1G>A was not present among the controls but c.93delG was found in two control subjects aged 37 and 55 years. The c.93delG mutation was further found among two unselected breast cancer patients from Tampere region of Finland, no c.837+1G>A mutations were found among this additional series.

In the screening of the unselected ovarian cancer patients by sequencing the exon 5 for the c.837+1G>A mutation, also the missense variant c.790G>A (p.Gly264Ser) was found from four subjects. The same variant had also been previously detected by Meindl *et al*. (33) both in cases and population controls. One of these ovarian cancer patients was also affected with breast cancer and another one had a mother affected with ovarian cancer, but the other two had no known family history of the disease. Further genotyping in all the patient series identified the variant also among breast cancer cases (0.5%) and in 0.2% of the population controls (Supplementary Material, Table S1). The frequency for the missense variant was not significantly different for any of the subgroups compared with population controls, however, there was a tendency for a higher frequency among ovarian cancer cases (1%, $P = 0.066$).

Frequencies of *RAD51C* mutations by patient series and family history

Combining both stages of the mutation testing, the frequencies of the *RAD51C* mutations in different subgroups and the risk ratios are shown in Table 3. The c.93delG was found in two out of 1279 (0.2%) in the population controls from Helsinki, but not among the 807 population controls from Tampere, the c.837+1G>A was not found in either control series. Altogether, no *RAD51C* mutations were found among sporadic breast cancer cases, breast cancer cases unselected for family history or familial breast cancer cases with history of breast but no ovarian cancer in the Helsinki series. Two carriers of the c.93delG were identified in the unselected breast cancer series from Tampere, without known family history of breast or ovarian cancer (two out of 686, 0.3%, $P = 0.211$ compared with population controls). All breast cancer cases combined (Helsinki and Tampere series), there were mutations in four out of 2239 (0.2%) breast cancer patients ($P = 0.7$ versus all population controls two out of 2086 (0.1%). Among the unselected breast cancer patients, the mutation frequency was 0.1% (two out of 1519, $P = 1$ versus controls). However, among the unselected ovarian cancer cases, the mutation carrier frequency was significantly higher than among population controls (1%, four out of 409, $P = 0.033$), two of the four mutation carriers did not have known family history of breast cancer.

When compared with cases by different family history in the Helsinki series, there were c.93delG or c.837+1G>A mutations in two out of 1553 (0.1%) breast cancer cases ($P = 1.0$ versus controls), both of whom had a strong family history of breast and ovarian cancer ($P = 0.004$ versus breast cancer cases without breast and ovarian cancer family history). When compared with population controls, the mutations associated with a high risk of familial breast and ovarian cancer (OR: 13.59, 95% CI 1.89–97.6, $P = 0.026$), but especially of familial ovarian cancer in the absence of breast cancer (OR: 213, 95% CI 25.6–1769, $P = 0.0002$) and also with an increased risk of unselected ovarian cancer (OR: 6.31, 95% CI 1.15–34.6, $P = 0.033$). Among all ovarian cancer patients, the prevalence was 1.4% (6 out of 417; OR: 9.37, 95% CI 1.87–46.4, $P = 0.004$ versus controls), with a significantly higher mutation rate among ovarian cancer cases with a family history of ovarian cancer [two out of eight = 25%, OR: 33.8, 95% CI 5.15–221, $P = 0.005$ versus ovarian cancer patients unselected for family history (4 out of 409 = 1%)]. No mutations were found among cases with familial breast cancer only and four out of the eight mutation carriers were ovarian cancer cases without family history of breast cancer.

Table 3. Frequencies of *RAD51C* mutations in different patient subgroups by family history and the risk ratios

	Total	wt	%	mut	%	*P*-value[1]	OR[1]	95% CI[1]	*P*-value[2]	OR[2]	95% CI[2]
RAD51C c.93delG											
Control	1279	1277	99.8	2	0.2						
All BC cases	1553	1552	99.9	1	0.1	0.592	0.41	0.04–4.54			
Familial BC	340	340	100	0	0.0	1					
Familial BC + OC	96	95	99.0	1	1.0	0.195	6.72	0.60–74.8	0.064		
Two affected BC	463	463	100	0	0.0	1					
Two affected BC + OC	62	62	100	0	0.0	1					
Sporadic BC	592	592	100	0	0.0	1					
Unselected BC	833	833	100	0	0.0	0.522					
All OC cases	417	414	99.3	3	0.7	0.099	4.63	0.77–27.8			
Familial OC	8	7	87.5	1	12.5	0.019	91.2	7.39–1126	0.057	29.1	2.35–359
Unselected OC	409	407	99.5	2	0.5	0.249	3.14	0.44–22.4			
Any OC	575	571	99.3	4	0.7	0.078	4.47	0.82–24.5			
Tampere-Control	807	807	100	0	0.0						
Tampere-BC	686	684	99.7	2	0.3	0.211					
RAD51C c.837+1G>A											
Control	1279	1279	100	0	0.0						
All BC cases	1553	1552	99.9	1	0.1	1					
Familial BC	340	340	100	0	0.0	1					
Familial BC + OC	96	95	99.0	1	1.0	0.070			0.064		
Two affected BC	463	463	100	0	0.0	1					
Two affected BC + OC	62	62	100	0	0.0	1					
Sporadic BC	592	592	100	0	0.0	1					
Unselected BC	833	833	100	0	0.0	1					
All OC cases	417	414	99.3	3	0.7	0.015					
Familial OC	8	7	87.5	1	12.5	0.006			0.057	29.1	2.35–359
Unselected OC	409	407	99.5	2	0.5	0.059					
Any OC	575	571	99.3	4	0.7	0.009					
RAD51C c.93delG or c.837+1G>A											
Control	1279	1277	99.8	2	0.2						
All BC cases	1553	1551	99.9	2	0.1	1					
Familial BC	340	340	100	0	0.0	1					
Familial BC + OC	96	94	97.9	2	2.1	0.026	13.59	1.89–97.6	0.004		
Two affected BC	463	463	100	0	0.0	1					
Two affected BC + OC	62	62	100	0	0.0	1					
Sporadic BC	592	592	100	0	0.0	1					
Unselected BC	833	833	100	0	0.0	0.522					
All OC cases	417	411	98.6	6	1.4	0.004	9.32	1.87–46.4			
Familial OC	8	6	75.0	2	25.0	0.0002	213	25.6–1769	0.005	33.8	5.15–221
Unselected OC	409	405	99.0	4	1.0	0.033	6.31	1.15–34.6			
Any OC	575	567	98.6	8	1.4	0.002	9.01	1.91–42.6			

Familial BC and Familial BC + OC include breast cancer cases with strong family history of breast cancer and breast and ovarian cancer, respectively. Two affected BC + OC includes breast cancer cases that have one first-degree relative affected with ovarian cancer and cases with both breast and ovarian cancer. Any OC includes all cases with personal or family history of ovarian cancer.

BC, breast cancer; OC, ovarian cancer; mut, mutation; wt, wild-type; *P*-value[1], *P*-value when compared with the controls; OR[1], odds ratio relative to controls; CI[1], confidence intervals relative to controls; *P*-value[2], OR[2], CI[2], *P*-value, odds ratio and confidence intervals relative to breast cancer patients without strong family history of breast and ovarian cancer or relative to unselected ovarian cancer patients.

Effects of the c.93delG and c.837+1G>A mutations

The single-nucleotide deletion c.93delG is predicted to be a protein truncating mutation and it leads to a premature stop codon in the first exon of *RAD51C* (p.Phe32SerfsX8). The splicing mutation c.837+1G>A disrupts the conserved 5′ splice donor site of exon 5. To further study the functionality of c.837+1G>A, we evaluated the effect of the mutation on splicing. We amplified the cDNA of two mutation carriers and one control subject using primers specific for *RAD51C* exons 3 and 8. Three different sized bands from c.837+1G>A mutation carriers were seen on agarose gel, but only one band from a control subject (Fig. 2A). Sequencing of the RT–PCR products confirmed that two mutant transcripts were present in the blood cells of the mutation carriers in addition to the wild-type transcript. The first mutant transcript lacks both exons 4 and 5 which disrupts the reading frame and leads to a premature stop codon (p.Glu191GlyfsX12) (Fig. 2D). The other mutant transcript lacks only exon 5 but does not affect the reading frame (p.Val236_Ala279del) (Fig. 2C). Only normal wild-type transcript was present in a control sample that was negative for the splicing mutation.

Haplotype analysis

To define whether the *RAD51C* mutation carrier families had a common ancestry, we performed haplotype analysis using seven SNP markers located on chromosome 17q adjacent to and within the *RAD51C* gene. The haplotype analysis revealed that mutation carriers in all the four families with the c.93delG

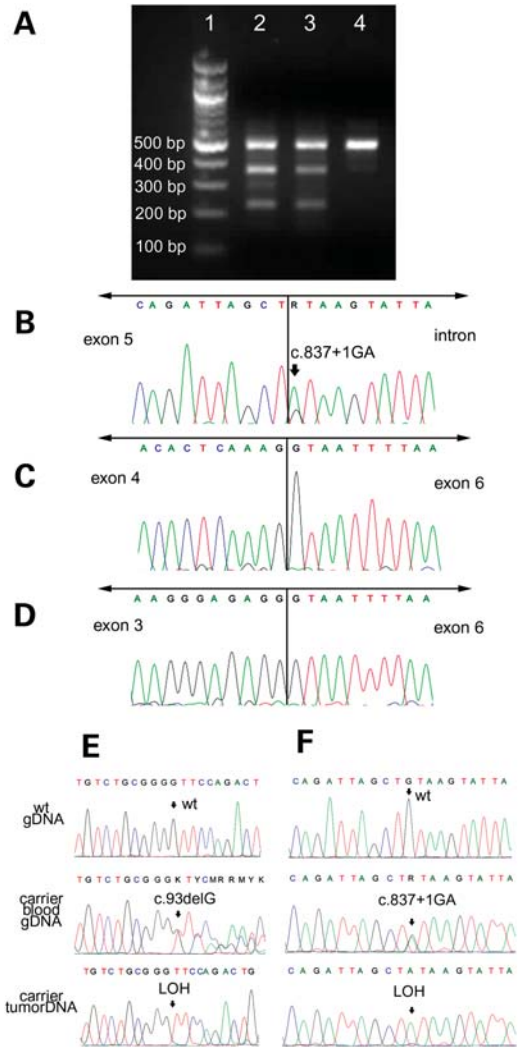

Figure 2. Splicing analysis of the c.837+1G>A mutation and LOH analysis of tumors from c.93delG and c.837+1G>A carriers. (**A**) Agarose-gel electrophoresis of RT–PCR products. Lane 1, 100 bp size standard; lane 2, cDNA of a c.837+1G>A mutation carrier; lane 3, cDNA of a c.837+1G>A mutation carrier; lane 4, cDNA of a control subject. (**B**) Genomic sequence of a c. 837+1G>A carrier. (**C**) cDNA sequence of the c. 837+1G>A carrier lacking exon 5. (**D**) cDNA sequence of the c. 837+1G>A carrier lacking exons 4 and 5. (**E**) Genomic sequences from wt and c.93delG mutation carrier and tumor DNA sequence from c.93delG carrier displaying LOH. (**F**) Genomic sequences from wt and c.837+1G>A mutation carrier and tumor DNA sequence from c.837+1G>A carrier displaying LOH.

mutation shared a common haplotype. Three c.837+1G>A carrier families with available genomic blood DNA samples were included in the haplotype analysis and mutation carriers in all these families shared a common haplotype as well.

Tumors of *RAD51C* mutation carriers

To find out whether there is loss of heterozygosity in the tumors of *RAD51C* mutation carriers, we sequenced exons 1 and 5 from DNA isolated from the tumors (Fig. 2E and F). We studied two breast and two ovarian tumors from c.847+1G>A mutation carriers and one breast tumor and four ovarian tumors from the c.93delG mutation carriers. All the ovarian tumors (six out of six) displayed LOH (loss of the wt allele) and two out of three breast tumors displayed LOH. The only tumor that did not show loss of heterozygosity was a breast tumor of c. 837+1G>A mutation carrier.

The clinicopathological features of the breast and ovarian tumors from the *RAD51C* mutation carriers are shown in Table 4. The mean age at first breast cancer diagnosis was 60.1 years among five patients. None of the breast tumors presented lymph-node infiltration or distant metastasis. The tumors were high grade with three tumors being grade 3 and one tumor grade 2. Three tumors were ductal and two lobular. All the tumors with available information were progesterone receptor negative but there was no consistency for estrogen receptor, p53 or HER2 status. The mean age at ovarian cancer diagnosis was 57.7 years among seven patients. Six tumors were serous ovarian carcinomas and one tumor was endometrioid. Six patients had developed a distant metastasis and only one patient was metastasis negative.

DISCUSSION

We have identified two recurrent pathogenic mutations in the *RAD51C* gene in Finnish breast and ovarian cancer patient series and further analyses in breast and ovarian cancer patients and families suggest *RAD51C* as a novel moderate-to-high penetrance ovarian cancer susceptibility gene. Both mutations are very rare in the population. The truncating frameshift deletion c.93delG had a 0.2% population carrier frequency among Helsinki region controls but was not found among controls from Tampere region, the splicing mutation c.837+1G>A was not present among the population controls at all. No mutations were found among breast cancer cases unselected for family history or among familial breast cancer cases with history of breast but no ovarian cancer in the Helsinki series, two sporadic carriers of the c.93delG were identified in the unselected breast cancer series from Tampere. Overall, the mutation frequency among breast cancer cases was not different from the population carrier frequency.

Our results from the several studied patient series of unselected and familial breast cancer and ovarian cancer cases as well as population controls indicate that *RAD51C* mutations do not confer a significantly increased risk of breast cancer *per se*, i.e. in the absence of ovarian cancer family history. The mutations associated with breast cancer cases only in the context of ovarian cancer family history (OR: 13.59, 95% CI 1.89–97.6, $P = 0.026$ compared with controls), with mutations found in about 2% of breast and ovarian cancer families. On the contrary, however, there was a high risk of familial ovarian cancer in the absence of breast cancer, the odds ratio for familial ovarian cancer patients in the absence of breast cancer family history was OR: 213 (95% CI 25.6–1769, $P = 0.0002$). Among all ovarian cancer patients (familial and unselected), the mutations were found in 1.4% of the cases (OR: 9.32, 95% CI 1.87–46.4, $P = 0.004$), with the highest prevalence among familial ovarian cancer cases in the absence of breast cancer family history (25 versus 1% among unselected ovarian cancer cases, OR: 33.8, 95% CI

Table 4. The tumor features of *RAD51C* mutation carriers

Mutation	LOH	Cancer	Dg age	T	M	N	Grade	Stage	Tumor histology	ER status	PR status	HER2 status	p53 status
c.837+1G>A	no	Breast	61.9	T1	neg	neg		1	Ductal				
c.837+1G>A	yes	Breast	45.0	T2	neg	neg	3	2A	Ductal	neg	neg	neg	pos
c.93delG	yes	Breast	54.7	T1	neg	neg	3	1	Ductal	neg	neg	pos	neg
c.93delG		Breast	76	T1	neg	neg	2	1	Lobular	pos	neg	neg	
c.93delG		Breast	63.1	T1	neg	neg	3		Lobular				
c.93delG	yes	Ovary	59		pos			1C	Serous				
c.93delG	yes	Ovary	60		pos	neg	1	3C	Serous				
c.837+1G>A	yes	Ovary	53		pos		3	3C	Serous				
c.837+1G>A	yes	Ovary	68		pos	pos	2	3C	Serous				
c.837+1G>A		Ovary	50		neg	neg	2	1A	Endometrioid				
c.93delG	yes	Ovary	60		pos	pos	3	3C	Serous				
c.93delG	yes	Ovary	54		pos	pos	3	4	Serous				

LOH, loss of heterozygosity; Dg age, diagnosis age; T, tumor size; M, metastasis; N, nodal status; ER, estrogen receptor; PR, progesterone receptor; neg, negative; pos, positive.

5.15–221, $P = 0.005$). In the unselected ovarian cancer series, *RAD51C* mutations conferred about 6-fold increased risk (OR: 6.31, 95% CI 1.15–34.6, $P = 0.033$); half of the mutation carriers did not have family history of breast cancer.

These results suggest that *RAD51C* is primarily a susceptibility gene for ovarian cancer and may contribute to a substantial proportion of familial ovarian cancer. The association with familial breast and ovarian cancer appears to be driven by the high risk of ovarian cancer. This is different from the *BRCA1* mutations, for instance, which confer an increased risk both for familial breast cancer and for familial ovarian cancer (6). Increased risk for breast cancer only in the context of ovarian cancer family history might indicate also interactive effects of the *RAD51C* mutations with other, so far unknown risk modifier alleles in other genes in such families.

Despite the rarity of the mutations and thus small numbers, the higher frequencies of mutations among cases with ovarian cancer family history suggest a significantly elevated familial ovarian cancer risk which may warrant consideration of genetic testing for the *RAD51C* mutations in Finnish cancer families where no *BRCA1* or *BRCA2* mutations have been found, and more careful pre-symptomatic screening or preventive measures for the carriers. However, more extensive studies on the penetrance of *RAD51C* mutations will be necessary for accurate evaluation of the carrier risks.

In the mutation carrier families, additional genotyping on available affected relatives provided some limited evidence of co-segregation with breast/ovarian cancer. All the breast and ovarian cancer patients in the mutation carrier families with DNA sample available were mutation positive. In family 1, the mother of the breast cancer proband also had breast cancer and shared her daughter's c.837+1G>A mutation (Fig. 1A). In family 5, the proband had breast cancer; one paternal cousin had breast cancer and another had breast and ovarian cancer; both shared her c.93delG mutation, although their mother also carried this mutation but had neither breast nor ovarian cancer (diagnosed with skin cancer at age 85) (Fig. 1D). Based on Mendelian sharing, the probability that all three tested breast/ovarian cancer relatives of carrier probands would be carriers by chance is 1 out of 32, i.e. 0.031 (over both families). The mutation carriers had a diverse cancer family history including also other cancers, for instance pancreatic cancer that is associated with *PALB2* mutations (37). Further studies for *RAD51C* mutations in patient series with other cancer types are warranted.

We also detected one missense variant c.790G>A (p.Gly264Ser) that had shown some mild evidence of association with familial breast and ovarian cancer in the study by Meindl *et al*. (33). Nevertheless, in our study, the variant had no statistical significance in any of the cancer patient groups when compared with population controls although there was a tendency for a higher frequency among ovarian cancer cases.

The mean age at first breast cancer diagnosis among the *RAD51C* mutation carriers was high (60.1 years) compared with the mean age at first breast cancer diagnosis for *BRCA1* or *BRCA2* mutation carriers (45.2 and 47.7 years, respectively) and also somewhat higher than among sporadic or familial, *BRCA1/BRCA2/PALB2* negative Finnish breast cancer patients (58.0 and 54.8 years, respectively) (38). For ovarian cancer diagnosis, the mean age was also higher (57.7 years) than for *BRCA1* mutation carriers but lower than among the *BRCA2* mutation carriers (51 and 61 years, respectively) (39) or in the general population (62 years) (40). In the study by Meindl *et al*. (33), the mean age at breast cancer diagnosis was lower than in our study: 53 years, but the mean age at ovarian cancer diagnosis was higher: 60 years.

With the limited histopathological data, it is difficult to estimate whether the *RAD51C* mutations confer a specific tumor phenotype. The breast tumor phenotype of carriers in this study was overall similar to that in the German families, with mostly ductal histology and poorly differentiated tumors (grades 2–3) but no consistency for hormone-receptor status. Six ovarian cancers were serous carcinomas and one was endometrioid, similar again to the German study. One of the unselected ovarian cancer patients with c.93delG mutation had fallopian tube carcinoma. However, recent studies have shown evidence that a great proportion of the serous ovarian carcinomas originate from fallopian tube especially in women with inherited *BRCA* mutations (41).

Both detected mutations lead to a truncated RAD51C protein. The single-nucleotide deletion c.93delG leads to a premature stop codon in the first exon of *RAD51C*. The splicing mutation c.837+1G>A disrupts the conserved 5′ splice donor site of exon 5 and leads to the skipping of previous exon(s), resulting in the formation of two mutant transcripts that were well detectable. The first mutant transcript lacks both exons 4 and 5 which disrupts the reading frame and leads to a premature stop codon in exon 6. The other mutant transcript lacks only exon 5 but does not affect the reading frame. There was loss of the wild-type allele in all ovarian tumors from the *RAD51C* mutation carries studied, in line with a tumor suppressor function for the *RAD51C* gene and supporting the deleterious effect of the mutations. Only one breast tumor from a c.837+1G>A carrier did not show evidence of loss-of-heterozygosity, whereas two other breast tumors had lost the wild-type copy.

In the Finnish population, *RAD51C* mutations are rare but in other populations they seem to be even more infrequent. Romero *et al*. (42) found one pathogenic *RAD51C* mutation (c.774delT) among 492 familial breast/ovarian cancer patients, whereas Zheng *et al*. (43) or Akbari *et al*. (44) did not identify any deleterious *RAD51C* mutations in patients with familial breast/ovarian cancer from different ethnic groups. *RAD51C* mutations were not found in male breast cancer patients either (45). The founder effects present in isolated populations including Finland (46,47) may lead to specific mutations becoming more common and explaining a major fraction of all mutations in specific genes. Such strong founder mutations have been identified in *BRCA1* and *BRCA2* genes in the Ashkenazi Jewish, Icelandic, Hungarian and also in the Finnish population (39,48–51). Unlike in the study of German breast cancer families by Meindl *et al*. (33) where six unique mutations were detected, two recurring pathogenic mutations were found in eight families in our study. According to haplotype analysis it is likely that these mutations are founder mutations which may be exclusively present in the Finnish population and that the families with mutation have a common ancestry.

Previously, recurrent, moderate penetrance mutations have been detected in the Finnish breast cancer families in the *PALB2* gene (38,52) and also in the *CHEK2* gene (53) that has a major role in the regulation of cellular responses to DNA double-strand breaks and assembly of the mitotic spindle (54). The frequency of *RAD51C* mutations among patients with family history of both breast and ovarian cancer (2%) is comparable to the frequency of *PALB2* variant in breast cancer families but lower than the frequency of *CHEK2* variant. The *PALB2* founder mutation c.1592delT was found in 2.0% of familial breast cancer cases (38). The frequency of the variant increases with increasing family history, being highest for patients with the strongest breast cancer family history (OR relative to controls, 14.27). The risk of breast cancer for c.1592delT carriers is estimated to be ∼40% by the age of 70 years (55). A *CHEK2* variant c.1100delC has been found in 5.5% of the familial breast cancer patients in the Finnish population, with about 4-fold elevated risk for familial breast cancer (53) and a 2-fold increase in risk also among women unselected for family history (56).

In conclusion, we have identified two recurrent, founder mutations in the *RAD51C* gene in the Finnish breast and/or ovarian cancer families. The mutation frequency among breast cancer cases and families was not different from the frequency in the population; however, it was significantly higher among the unselected ovarian cancer cases than among population controls and further, *RAD51C* mutations were ∼25 times more frequent among ovarian cancer cases with an ovarian cancer family history but no breast cancer family history than among cases unselected for family history. These results indicate *RAD51C* as a novel moderate-to-high risk susceptibility gene for ovarian cancer with also clinical significance for diagnostic testing and more careful presymptomatic screening or preventive measures for the carriers. Although rare, the *RAD51C* mutations may contribute to a small fraction of families including both breast and ovarian cancer cases but possibly a larger fraction of ovarian cancer families, and further studies on ovarian cancer families in other populations are warranted.

MATERIALS AND METHODS

Subjects

The *RAD51C* gene was screened for germline variation in 277 familial *BRCA1/2* negative breast or ovarian cancer patients from Helsinki region. Altogether, 130 of the patients belong to families with multiple breast cancer cases, 139 in families with both breast and ovarian cancer and 8 in ovarian cancer families. All the patients from breast cancer families and 94 patients from breast and ovarian cancer families had strong familial background with three or more breast or ovarian cancers among first- or second-degree relatives including the proband. Forty-five patients with breast and/or ovarian cancer background were from families with two first-degree relatives affected with breast or ovarian cancer.

The detected *RAD51C* mutations c.93delG and c.837+1G>A and the variant c.790G>A were further screened in an unselected series of 884 breast cancer patients, in 491 additional familial breast cancer patients, 409 ovarian cancer patients and 1279 healthy female population controls from the same geographic region. Two hundred and twelve of these cases had strong familial background of breast or breast and ovarian cancer, 463 were familial cases with one first-degree relative and the proband affected with breast cancer and 17 cases were affected with both breast and ovarian cancer. Five hundred and ninety-two patients had sporadic breast cancer. The ovarian cancer patients were unselected for family history.

The mutations c.93delG and c.837+1G>A were also further screened in 686 breast cancer patients and 807 healthy population controls from Tampere region of Finland (57).

The collection of the patient series and data is described in detail in the Supplementary Material.

This study was carried out with informed consent from all the patients and with permission from the Ethics Committee of the Helsinki University Central Hospital and from the Ministry of Social Affairs and Health in Finland.

Mutation screening

The coding region, the exon–intron boundaries and the 5′UTR of the *RAD51C* gene were screened on genomic DNA isolated from blood samples of 277 patients with bidirectional sequencing using ABI BigDyeTerminator 3.1 Cycle Sequencing kit (Applied Biosystems). Primers and PCR conditions for amplification of *RAD51C* exons are presented in Supplementary Material, Table S2. The sequencing results were analyzed with Variant Reporter Software v1.0 (Applied Biosystems).

Genotyping

We further genotyped 884 unselected breast cancer patients, 491 additional familial breast cancer patients, 409 ovarian cancer patients and 1279 healthy population controls for the two detected *RAD51C* mutations c.93delG and c.837+1G>A and the c.790G>A variant. The c.93delG and c.837+1G>A mutations were also genotyped in 686 breast cancer patients and 807 healthy population controls from Tampere region of Finland. For all other samples, genomic DNA was isolated from blood, whereas 80 samples from the ovarian cancer patients were tumor DNA. The breast cancer patients and the controls, as well as 80 tumor DNA samples from the ovarian cancer patients, were genotyped using TaqMan Custom Assays specific for mutations c.93delG and c.837+1G>A and TaqMan Genotyping Master Mix (Applied Biosystems). PCR was performed in 7500 Fast Real-Time PCR System (Applied Biosystems) or in 9800 Fast Thermal Cycler (Applied Biosystems) and genotype calling was performed with 7500 Fast Real-Time PCR System and ABI Prism 7500 SDS v1.4 software (Applied Biosystems). Screening of the mutations in 329 ovarian cancer patients was done by unidirectional sequencing using primers specific for *RAD51C* exons 1 and 5 (Supplementary Material, Table S2). Additional genotyping was also done in relatives of the mutation carriers with available DNA samples with bidirectional sequencing using primers specific for exon 1 or 5.

RNA extraction and splicing analysis

Blood samples from the subjects in the splicing analysis of the c.837+1G>A mutation were collected into PAXgene Blood RNA tubes and the total RNA was isolated using PAXgene Blood RNA kit according to the manufacturer's protocol (PreAnalytiX). Total RNA was converted into single-stranded cDNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. RT–PCR was performed with Amplitaq Gold Polymerase (Applied Biosystems) using primers specific for *RAD51C* exons 3 and 8 (Supplementary Material, Table S2). The PCR products were analyzed on 2% agarose gel and extracted with MinElute Gel Extraction kit (Qiagen) and sequenced after extraction. cDNA from a control subject negative for the *RAD51C* splicing mutation was amplified with RT–PCR and sequenced directly after the amplification without gel extraction.

Loss-of-heterozygosity analysis

Representative areas of tumor tissue in paraffin-embedded samples were identified by an experienced breast cancer pathologist. We punched tissue cores from paraffin-embedded tumor tissues with manual tissue arrayer needle and extracted DNA with standard phenol–chloroform method. We amplified the DNA using primers specific for *RAD51C* exon 1 or 5 (Supplementary Material, Table S3) and sequenced the PCR-products. The sequencing results of the tumor samples were compared with heterozygous germline DNA isolated from the blood.

Haplotype analysis

We used seven SNP markers located at positions 17:56768103–17:56802458 spanning 34 355 bp to define whether the *RAD51C* mutation carrier families had a common ancestry. SNPs rs12946522 (first intron of *TEX14*), rs17222691 (first intron of *RAD51C*), rs9916423 (second intron of *RAD51C*), rs28363318 (sixth intron of *RAD51C*) as well as rs304269, rs304270 and rs304271 (seventh intron of *RAD51C*) were genotyped with sequencing in 14 subjects from seven mutation carrier families.

Statistical analyses

The statistical analyses were performed using SPSS 15.0 for Windows (SPSS, Inc.). All *P*-values are two-sided and calculated using Fischer's exact test.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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