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EPIDEMIOLOGY

Further evidence for the contribution of the *RAD51C* gene in hereditary breast and ovarian cancer susceptibility

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Abstract RAD51C, a RAD51 paralogue involved in homologous recombination, is a recently established Fanconi anemia and breast cancer predisposing factor. In the initial report, *RAD51C* mutations were shown to confer a high risk for both breast and ovarian tumors, but most of the replication studies published so far have failed to identify any additional susceptibility alleles. Here, we report a full mutation screening of the *RAD51C* gene in 147 Finnish familial breast cancer cases and in 232 unselected ovarian cancer cases originating from Finland and Sweden. In addition, in order to resolve whether common *RAD51C* SNPs are risk factors for breast cancer, we genotyped five tagging single nucleotide polymorphisms, rs12946522, rs304270, rs304283, rs17222691, and rs28363312, all located within the gene, from 993 Finnish breast cancer cases and 871 controls for cancer associated variants. Whereas, none of the studied common SNPs associated with breast cancer susceptibility, mutation analysis revealed two clearly pathogenic alterations. *RAD51C* c.-13_14del27 was observed in one familial breast cancer case and c.774delT in one unselected ovarian cancer case, thus confirming that *RAD51C* mutations are implicated in breast and ovarian cancer predisposition, although their overall frequency seems to be low. Independent identification of the very recently reported *RAD51C* c.774delT mutation in yet another patient originating from Sweden suggests that it might be a recurrent mutation in that population and should be studied further. The reliable estimation of the clinical implications of

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carrying a defective *RAD51C* allele still requires the identification of additional mutation positive families.

Keywords *RAD51C* · Hereditary breast and ovarian cancer susceptibility · Germline mutation

Introduction

The presence of a family history is the most important risk factor for the development of breast cancer, with the first-degree relatives having approximately a twofold increased risk over the general population. However, <25% of familial breast cancer is explained by inherited mutations in the known susceptibility genes, such as *BRCA1*, *BRCA2*, and *PALB2* [1, 2]. The rest of the cases are suggested to be explained by common low risk variants, although the data from large multiple case families strongly suggest that there might still be a few additional high penetrance genes to be identified [1]. Most of the genes connected to familial breast cancer are involved in the repair of DNA double-strand breaks through homologous recombination (HR) [3]. Mutations in three of these genes, *BRCA2*, *BRIP1* and *PALB2*, are also associated with Fanconi anemia (FA) [4, 5], establishing a strong link between breast cancer susceptibility and the FA pathway. This link is further reinforced by the identification of the newest player *RAD51C*, a RAD51 paralogue involved in HR, in both of these hereditary conditions. Biallelic *RAD51C* mutations have recently been identified in FA patients (FANCO) and subsequently monoallelic mutations have been found to confer an increased risk for breast cancer [6, 7].

RAD51C is part of a machinery composed of altogether five RAD51 paralogues (RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3) that ensures the timely and precise loading of RAD51, the key protein in the HR pathway mediating homologous pairing of DNA sequences and strand exchange, at the sites of DNA damage. The RAD51 paralogues interact with each other and form two different subcomplexes. RAD51C is thought to play a central role in these associations, as it is the only one of the paralogues which is part of both complexes [8, 9]. In addition to promoting RAD51 assembly, RAD51C is important for processing HR intermediates late in the DNA repair as it participates in branch migration and resolving of Holliday junctions [10]. From the FA network perspective, RAD51C is located downstream of the classical part of the pathway, the core FA complex (FANCA, -B, -C, -E, -F, -G, -L, and -M proteins) and the I-D2 complex (FANCD2 and -I proteins), which is analogous to BRCA2 (FANCD1), BRIP1 (FANCJ), and PALB2 (FANCN) [6, 11]. RAD51C defective cells show impaired RAD51 foci formation after DNA damage and are hypersensitive to DNA cross-linking agents, which is a hallmark of FA. The clinical phenotype of *RAD51C* defected patients, however, differs in some features from the other mutations in this part of the pathway, as the patients lack hematological abnormalities and malignancies. The affected family members do show some of the severe congenital abnormalities, such as imperforate anus and cystic kidneys with renal failure, also described in *BRCA2* and *PALB2* defective FA patients [6].

Being among the most recently identified genes in the downstream part of the FA pathway, it is conceivable that monoallelic mutations in *RAD51C* could explain some of the *BRCA1*/*BRCA2* mutation negative breast cancer families. Indeed, six monoallelic dominant *RAD51C* mutations were subsequently identified in 480 German families displaying both breast and ovarian tumors. Curiously, *RAD51C* mutations were not observed in families with breast cancer only [7]. As regards ovarian cancer occurrence the *RAD51C* families thus show major similarities with the families carrying *BRCA1* and *BRCA2* mutations, whereas families segregating mutations in the *PALB2* and *BRIP1* genes lack this feature [2, 12]. Moreover, as the *RAD51C* families showed apparently complete segregation of the mutation with the cancer phenotype, the penetrance of the *RAD51C* mutations was predicted to be at least comparable to that of *BRCA1* and *BRCA2* mutations. In order to get more information on *RAD51C*'s contribution to cancer susceptibility, we performed a sequence analysis of the gene for germline mutations in 147 *BRCA1/2* mutation negative familial breast cancer cases and in 232 ovarian cancer patients unselected for a family history of cancer, and genotyped five tagging single nucleotide polymorphisms (tagSNPs) from 993 breast cancer cases and 871 controls for cancer associated variants in the *RAD51C* gene region. Two deleterious *RAD51C* mutations, c.-13_14del27 and c.774delT, were identified, thus confirming the contribution of *RAD51C* defects to breast and ovarian cancer susceptibility.

Materials and methods

Patients and controls

Affected index cases of 147 breast, or breast and ovarian cancer families were sequenced for *RAD51C* mutations. All these families originated from Northern Finland and were negative for Finnish *BRCA1/2* and *PALB2* founder mutations. The tested 11 *BRCA1/2* founder mutations account for the majority of all mutations in these genes in Finnish population [2, 13]. Ninety-nine of the families were considered as high risk families: 74 of these families had three or more cases of breast, or breast and ovarian cancer in first- or second-degree relatives and 25 had two

cases of breast, or breast and ovarian cancer in first- or second-degree relatives, of which at least one had early disease onset (≤35 years), bilateral breast cancer, or multiple primary tumors including breast or ovarian cancer. Forty-eight of studied families displayed moderate disease susceptibility with two cases of breast, or breast and ovarian cancer in first- or second-degree relatives, or one case showing strong indication of hereditary predisposition to disease (multiple primary tumors including breast cancer at young age, altogether four index cases). Of the studied 147 families, 112 (76.2%) were with breast cancer only, and 35 families (23.8%) showed both breast and ovarian cancer. All of the patient samples have been collected at the Oulu University Hospital with their informed consent.

An additional breast cancer cohort of altogether 993 patients unselected for family history of cancer was used for the c.-13_14del27 and T287A genotyping, and tagSNP analysis. This cohort consisted of 542 Northern Finnish breast cancer cases diagnosed at the Oulu University Hospital between the years 2000 and 2007, and of 451 prospective breast cancer cases from the province of Northern Savo in Eastern Finland, diagnosed at the Kuopio University Hospital between April 1990 and December 1995 [14]. A total of 871 Finnish healthy females were used as controls. Of these 507 were anonymous cancer-free female Northern Finnish Red Cross blood donors (age ≥45 years), and 364 were control subjects from the province of Northern Savo in Eastern Finland, who were age and area-of-residence matched, selected from the National Population Register during the same time period as the Kuopio unselected breast cancer patient material. The presence of the c.-13_14del27 allele was also tested in an unselected ovarian cancer cohort of 332 patients diagnosed at the Oulu University Hospital.

For the confirmation of *RAD51C* contribution to ovarian cancer susceptibility, a cohort of 232 ovarian cancer patients unselected for a family history of the disease was comprehensively screened for mutations in this gene. The study set included 204 Swedish patients diagnosed at the Sahlgrenska University Hospital in Gothenburg, and 28 Finnish patients diagnosed at the Kuopio University Hospital. Approval to perform the study was obtained from the Ethical Boards of the involved University Hospital Health Care Districts.

RAD51C mutation analyses

Genomic sequencing (ABI3130xl Genetic Analyzer, Applied Biosystems, Carlsbad, CA) of 147 familial breast cancer cases and High-Resolution Melt (HRM) analysis (CFX96, Bio-Rad, Hercules, CA) of the 232 unselected ovarian cancer cases was performed based on sequence information (NC_000017.10, NM_058216.1) obtained from publicly available databases. Intronic primers were designed using Primer3 software. Homologous sequences were identified by BLAT search and these were avoided in the designing process. PCR primers and reaction conditions are available on request. All identified variants were confirmed by re-sequencing original DNA in both directions. Sequence data was analyzed using Codon Code Aligner (CodonCode Corporation, Dedham, MA) and MEGA4 [15] analysis software.

Study for the occurrence of two *RAD51C* sequence alterations c.-13_14del27 and T287A (rs28363317) observed in genomic sequencing as well as the genotyping of five tagSNPs (rs12946522, rs304270, rs304283, rs17222691, and rs28363312) for the association analysis was carried out in an unselected breast cancer patient cohort and in healthy female controls by using MassARRAY® (Sequenom Inc., San Diego, CA) and iPLEX® Gold (Sequenom, Inc.) on a 384-well plate format. TagSNPs were selected using the HapMap Genome Browser release 2 (Phase 3, NCBI build 36, bdSNP b126) as of May 28, 2010 (http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap3r2_B36/). TagSNPs for region chr17:54118611–54173040 were picked out for the CEU population using the Tagger multimarker algorithm with r^2 cutoff at 0.8 and minor allele frequency (MAF) cutoff at 0.05. MassARRAY mass spectrometer (Sequenom, Inc.) was used for spectra acquisitions from the SpetroCHIP. Data analysis and genotype calling were done using TyperAnalyzer Software version 4.0.3.18 (Sequenom, Inc.). Each 384-well plate contained a minimum of eight non-template controls. For the c.-13_14del27 change DNA from one heterozygous mutation carrier and for T287A DNA from two heterozygous carriers were used as positive controls on each plate. For quality control, duplicate analysis was done for 6.5% of the Oulu samples and for 6.7% of the Kuopio samples. All primer sequences are available on request.

Statistical analyses

Carrier frequencies between cases and controls were compared using Fisher's exact test (two-sided) (PASW Statistics 18.0 for Windows, SPSS Inc., Chicago, IL). For tagSNP data, the overall association as well as the Hardy-Weinberg equilibrium, allele specific P, OR, and CI were computed using Cochran-Armitage trend test.

Results

Sequencing of *RAD51C* in index cases of 147 breast, or breast–ovarian cancer families revealed two sequence alterations: one leading to a single amino acid change (T287A) and one to a 27 base pair (bp) deletion in exon 1

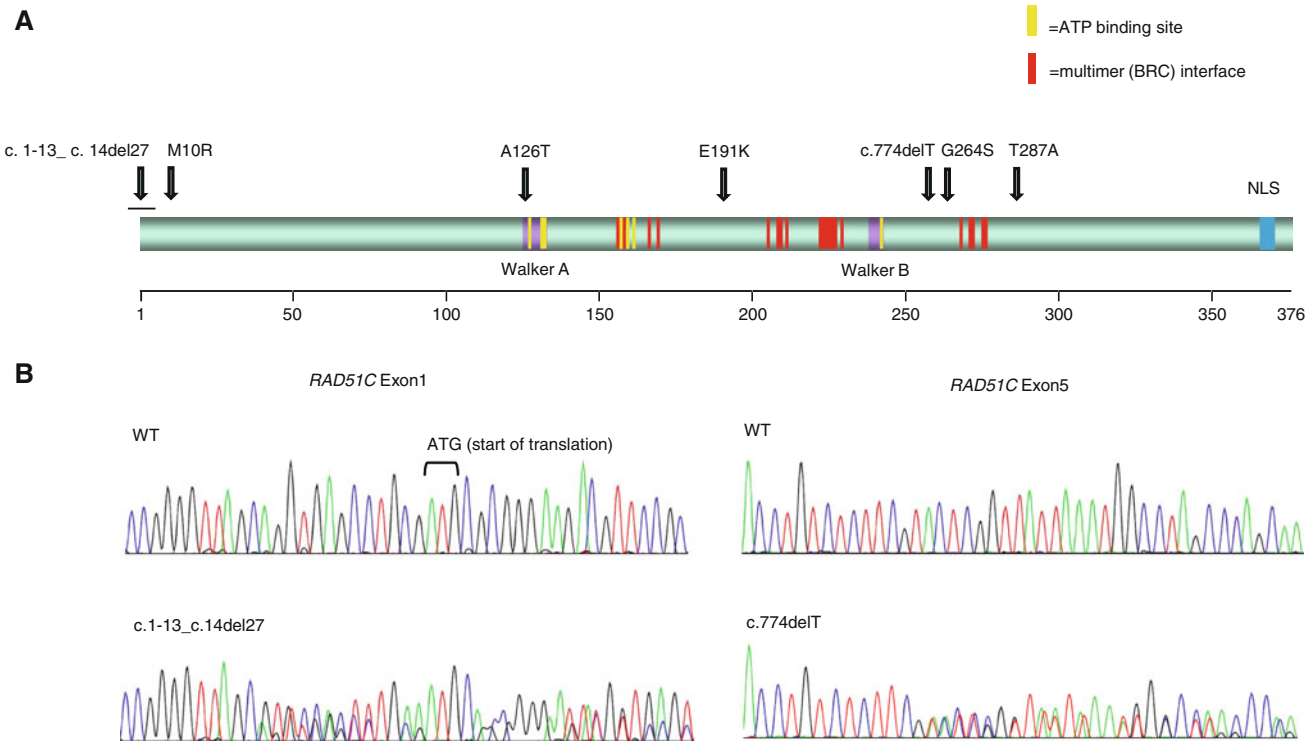

Fig. 1 **a** Schematic diagram of the RAD51C protein showing predicted functional domains and the sites of the observed sequence changes. Putative NLS (nuclear localization signal), (NP_478123.1, [23]). **b** Sequence characterization of the observed germline mutations c.-13_14del27 and c.774delT of *RAD51C*

Table 1 Observed sequence changes in the *RAD51C* gene in familial breast cancer patients

Location	Nucleotide change	Effect on protein	Novelty	Carrier frequency			*P* (OR; 95% CI)[b]
				Familial Br	Unselected Br	Controls	
Ex1	c.-13_14del27	No transcription[a]	Novel	1/147	0/990	0/852	0.147 (NA)
Ex6	c.859A>G	T287A	rs28363317	2/147	0/984	2/860	0.104 (5.9; 0.8–42.0)

Br breast cancer, *NA* not available, *OR* odds ratio, *CI* confidence interval

[a] Based on FPROM human promoter prediction software and HMM-based gene structure prediction software, FGENESH

[b] Fisher's Exact Test, familial Br cases vs. controls

(Fig. 1, Table 1). The T287A missense variant (rs28363317, [7]) was observed in two familial cancer index cases diagnosed with breast cancer, and was considered a benign alteration based on its prevalence in the healthy controls (2/860, $P = 0.104$) and absence from the unselected breast cancer cohort (0/984). The other alteration, c.-13_14del27 leading to a 27 bp deletion of the sequence around the translation initiation site, was observed in the index case of one family (Fig. 2). According to FPROM human promoter prediction software (Softberry, Inc., Mt. Kisco, NY) this deletion transfers the transcription start site 95 nucleotides downstream compared to that of the wild type allele, and all sequences needed for reliable predictions for this exon made by HMM-based gene structure prediction software, FGENESH (Softberry, Inc., Mt. Kisco, NY), are abolished. Based on bioinformatics, the observed 27 bp deletion thus produces a null allele, and is pathogenic. The index of the family with the *RAD51C* c.-13_14del27 allele was diagnosed with breast cancer at the age of 53 years, but she also developed leukemia 1 year after the first primary cancer. Her mother had breast cancer at the age of 69, and similarly, was also diagnosed with leukemia at the age of 76 years. Although this family showed very strong genetic predisposition to cancer, breast and ovarian in particular, the segregation of the mutation with the disease phenotype could not be demonstrated because of the lack of suitable specimens from deceased individuals. In addition to 852 healthy controls, the prevalence of *RAD51C* c.-13_14del27 was tested in

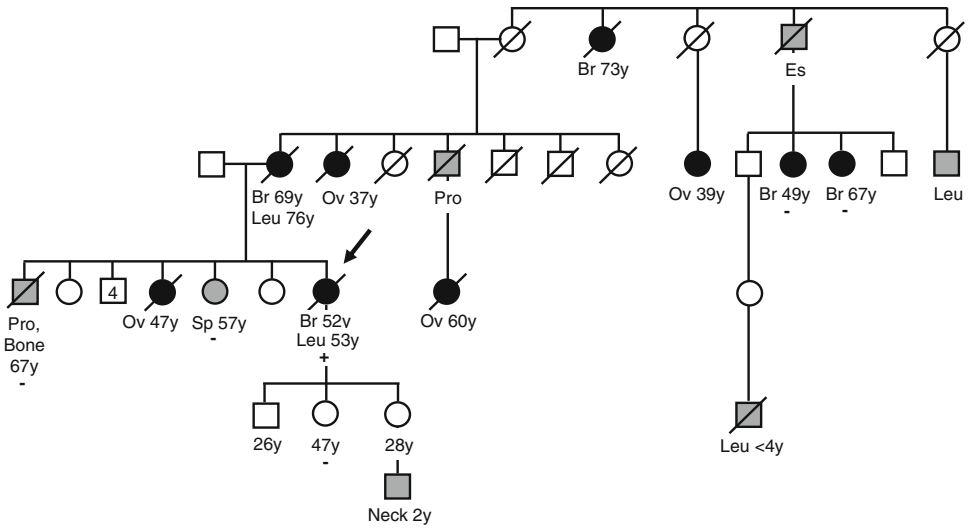

Fig. 2 Pedigree of breast cancer patient carrying the *RAD51C* c.-13_14del27 allele. *Black circles* represent patients with breast or ovarian cancer; other cancer types are marked with *gray*. *Arrow points* to the index patient. *Br* breast cancer, *Bone* bone cancer, *Es* esophagus cancer, *Leu* leukemia, *Ov* ovarian cancer, *Neck* neck tumor, *Pro* prostate cancer, *Sp* spinocellular carcinoma. If known, age at diagnosis or the time of monitoring is shown

990 unselected breast cancer cases and 332 ovarian cancer cases but no additional carriers were identified.

Besides screening the entire gene for rare deleterious mutations, we also investigated whether common SNPs in *RAD51C* associate with breast cancer susceptibility. The allele distribution of five *RAD51C* tagSNPs, rs12946522, rs304270, rs304283, rs17222691, and rs28363312, locating within the gene was compared between 993 unselected breast cancer cases and 871 geographically matched unaffected controls. There was no evidence of a statistically significant association for any of these tagSNPs with breast cancer risk, although two of them, rs12946522 and rs17222691, in strong linkage disequilibrium suggested a borderline significant association with cancer susceptibility—a finding that needs to be confirmed by further studies with larger sample set (Table 2).

Based on the strong indication that *RAD51C* mutations predispose not only for breast, but also for ovarian cancer, an additional ovarian cancer cohort ($n = 232$) unselected for a family history of the disease was comprehensively screened for *RAD51C* mutations by using the HRM prescreening method. This revealed several alterations (Fig. 1, Table 3): five amino acid changes, one intronic variant, and one frameshift mutation. All sequence alterations were observed in ovarian cancer patients originating from Sweden ($n = 204$). Three of the observed changes were novel ones and two of them, M10R and E191K, led to amino acid substitutions. Both affected residues showed evolutional conservation among mammals and zebra fish. The M10R change was observed in a patient diagnosed with ovarian cancer at the age of 80 years and was shown to have a deleterious effect on the protein by two different predictive algorithms. Also, E191K was considered possibly damaging by PolyPhen. It was observed in two index patients, both showing a clear indication for hereditary predisposition to cancer: the other carrier had been diagnosed with both breast and ovarian cancer at the age of 43 and 50 years, respectively, and the other was diagnosed with ovarian cancer at the age of 36 years. However, to reliably demonstrate the pathological nature of the M10R and E191K missense alterations, functional analyses would be required. Until then both remain missense alterations of uncertain clinical significance. Of the other four previously reported changes one, c.774delT, had a clearly deleterious effect on the protein as it led to a frameshift and protein truncation, and was considered pathogenic. The c.774delT carrier had been diagnosed with ovarian cancer at the age of 61 years. Both of her parents and also one sibling had been diagnosed with cancer but, unfortunately, no additional details of the type of cancer or of the age at malignancy diagnosis were available.

Table 2 Association of five *RAD51C* tagSNPs with breast cancer

TagSNP	Minor allele	MAF cases	MAF controls	*P* value[a]
rs12946522	G	0.26	0.23	0.071
rs304270	G	0.33	0.34	0.369
rs304283	T	0.27	0.26	0.944
rs17222691	T	0.28	0.25	0.078
rs28363312	A	0.09	0.09	0.969

[a] Cochran-Armitage trend test

Discussion

RAD51C is an integral part of the DNA double-strand break repair through HR, and was recently established as a FA and breast cancer predisposing factor [5]. This study provides further evidence that germline defects in *RAD51C* are implicated in cancer predisposition, as two clearly deleterious mutations, c.-13_14del27 and c.774delT, were

Table 3 Observed sequence changes in the *RAD51C* gene in unselected ovarian cancer patients

Location	Nucleotide change	Effect on protein	SIFT	PolyPhen	Novelty	N^a	MAF[b]	
							This study	CEU
Ex1	c.29T>G	M10R	Not tolerated	Probably damaging	Novel	1/232		–
Ex2	c.376G>A	A126T	Tolerated	Benign	rs61758784	2/232	0.004	n.d.[c]
Ex3	c.571G>A	E191K	Tolerated	Possibly damaging	Novel	2/232	0.004	–
IVS3	c.571-17G>T	Intronic[d]	–	–	Novel	2/232	0.004	–
Ex5	c.774delT	R258fs	–	–	[22]	1/232		–
Ex5	c.790G>A	G264S[e]	Tolerated	Benign	[7]	2/232	0.004	–
Ex6	c.859A>G	T287A	Tolerated	Probably damaging	rs28363317	4/232	0.009	0.017

Used predictive algorithms: NNSplice Software (http://www.fruitfly.org/seq_tools/splice.html), SIFT (http://sift.jcvi.org/), PolyPhen (http://genetics.bwh.harvard.edu/pph/)

[a] All changes observed in patients originating from Sweden ($n = 204$)

[b] Minor allele frequency not displayed for singletons

[c] No frequency data

[d] No effect on splicing consensus sequences (NNSplice Software), always observed together with c.376G>A

[e] Amino acid change G264S is suggested to be associated with malignancies in the subgroup of BC/OC families by Meindl et al. [7]

identified in the studied cohorts. In contrast, none of the common SNPs within *RAD51C* associated with cancer susceptibility, indicating that these are not major breast cancer risk factors, at least not in the studied population.

RAD51C c.-13_14del27 was observed in one of the 147 familial breast cancer cases. Similarly to the initial report by Meindl et al. [7], this family had a history of both breast and ovarian cancer. The *RAD51C* c.-13_14del27 family also had exceptionally high prevalence of leukemia cases, although the co-segregation of the *RAD51C* mutation with these could not be confirmed. The index herself developed leukemia 1 year after breast cancer diagnosis, suggesting a possible treatment association. Interestingly, a similar association with leukemia and treatment-associated leukemia has previously been suggested for *BRCA1* and *BRCA2* mutations [16]. The prevalence of *RAD51C* c.-13_14del27 mutation was also tested in 990 geographically matched breast and 332 ovarian cancer patients unselected for a family history of the disease, but additional carriers were not observed. This indicates that *RAD51C* c.-13_14del27 is a very rare, or even a private mutation rather than a common Finnish founder alteration that would explain a more significant fraction of breast or ovarian cancer susceptibility in our population.

Of the currently studied 147 families, 35 displayed both breast and ovarian cancer, and the frequency of *RAD51C* mutations in them (1/35, 2.9%) was comparable to that reported previously (6/480, 1.25%) [7], although the number of this subset of families in this study was relatively small. Correspondingly, no *RAD51C* mutations were observed in the families with breast cancer only, which underlines the connection between *RAD51C* defects and predisposition to ovarian cancer. This was further strengthened by the identification of another deleterious alteration, *RAD51C* c.774delT, in the subsequently analyzed ovarian cancer cohort of 232 patients unselected for a family history of the disease, the identified mutation carrier originating from Sweden. The screening of the ovarian cancer cohort also revealed two missense mutations, M10R and E191K, with potential clinical relevance, as they targeted evolutionally conserved residues and were predicted to have damaging effects on the protein.

The other replication studies published to date have, quite unexpectedly, failed to identify any clearly pathogenic mutations in *RAD51C* [17–21], with the exception of the study by Romero et al. [22] who reported a single deleterious *RAD51C* alteration in one Spanish breast and ovarian cancer family. Curiously, the reported alteration, c.774delT, was the same as independently observed in our study. To make things even more interesting, the Spanish mutation positive breast cancer patient inherited the c.774delT allele from her Swedish mother, who was still healthy at the age of 70 years. Altogether this data strongly suggests that c.774delT is a recurrent Swedish mutation, and should be studied further in this population. The identification of additional *RAD51C* mutation positive families would also provide the means for more reliable cancer risk assessments.

Although the initial report implied that *RAD51C* is a very promising candidate to fulfill the expectations set up for breast–ovarian cancer susceptibility gene 3 or "*BRCA3*" (as already referred in OMIM) in regards of disease penetrance and cancer spectrum, the current evidence [17–22, this study] indicate that mutations in this gene are significantly much rarer than those occurring in *BRCA1* and *BRCA2*. Overall, the results of the current *RAD51C* mutation analysis

and numerous previous studies [3, 7] further support the idea that rare gene variants, with either moderate or potentially even high disease penetrance, are important components of familial breast cancer. Indeed, virtually all breast cancer susceptibility genes identified to date are related to fundamental processes in cellular proliferation and DNA integrity control. The natural selection maintains the frequency of these alleles low as biallelic mutations in most of these genes have severe consequences, these individuals being either non-viable or having reduced fertility. It is likely that more of these rare breast cancer predisposing alleles will be found; the genes involved in the FA/BRCA pathway evidently being the most plausible candidates for harboring such mutations. It is possible that these, yet unidentified, rare alleles are limited in their geographical occurrence and each accounts only for a small fraction of the families. This will make their identification, and also subsequent confirmation of their pathological nature, even more challenging.

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Conflict of interest statement None.

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