IJC
International Journal of Cancer

About 1% of the breast and ovarian Spanish families testing negative for *BRCA1* and *BRCA2* are carriers of *RAD51D* pathogenic variants

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Cancer Genetics

RAD51D **mutations have been recently identified in breast (BC) and ovarian cancer (OC) families. Although an etiological role in OC appears to be present, the association of *RAD51D* mutations and BC risk is more unclear. We aimed to determine the prevalence of germline *RAD51D* mutations in Spanish BC/OC families negative for *BRCA1/BRCA2* mutations. We analyzed 842 index patients: 491 from BC/OC families, 171 BC families, 51 OC families and 129 patients without family history but with early-onset BC or OC or metachronous BC and OC. Mutation detection was performed with high-resolution melting, denaturing high-performance liquid chromatography or Sanger sequencing. Three mutations were found in four families with BC and OC cases (0.82%). Two were novel: c.1A>T (p.Met1?) and c.667+2_667+23del, leading to the exon 7 skipping and one previously described: c.674C>T (p.Arg232*). All were present in BC/OC families with only one OC. The c.667+2_667+23del cosegregated in the family with one early-onset BC and two bilateral BC cases. We also identified the c.629C>T (p.Ala210Val) variant, which was predicted *in silico* to be potentially pathogenic. About 1% of the BC and OC Spanish families negative for *BRCA1/BRCA2* are carriers of *RAD51D* mutations. The presence of several BC mutation carriers, albeit in the context of familial OC, suggests an increased risk for BC, which should be taken into account in the follow-up and early detection measures. *RAD51D* testing should be considered in clinical setting for families with BC and OC, irrespective of the number of OC cases in the family.**

Key words: *RAD51D*, genetic predisposition, familial breast and ovarian cancer
Additional Supporting Information may be found in the online version of this article.
Conflicts of interest: Nothing to report
Grant sponsor: Miguel Servet Program, Spanish Carlos III Health Institute; **Grant number:** CP10/00617; **Grant sponsor:** Fondo de Investigación Sanitaria (FIS) Research; **Grant number:** PI 12/00539; **Grant sponsor:** Intrasalud; **Grant number:** PI12/00070; **Grant sponsor:** Xunta de Galicia; **Grant number:** 10PXIB 9101297PR; **Grant sponsors:** Mutua Madrileña Foundation (FMMA); **Grant sponsor:** Acción Cooperativa y Colaborativa Intramural-CIBER2012, SAF2010-20493; **Grant sponsor:** Cancer Prevention Program of the Regional Government of Castilla y León
DOI: 10.1002/ijc.28540
History: Received 5 Aug 2013; Accepted 27 Sep 2013; Online 15 Oct 2013
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What's new?
RAD51D **mutations have recently been identified in breast (BC) and ovarian (OC) cancer families. Although** ***RAD51D*** **mutations are associated with hereditary OC, such an association is less clear in BC. This study determined the prevalence of germline** ***RAD51D*** **mutations in Spanish BC/OC families testing negative for** ***BRCA1/BRCA2*** **mutations.** ***RAD51D*** **mutations were found in almost 1% (4/491) of BC/OC families. These families had only one OC case, with some carriers presenting BC, suggesting that** ***RAD51D*** **testing should be offered to all BC/OC families. The existence of an increased risk of BC should be considered when setting the follow-up and prevention measures.**

Ovarian cancer (OC) represents the eighth most commonly diagnosed cancer among women in the world, and causes more deaths per year than any other cancer of the female reproductive system.[1] On a worldwide basis, 224,747 new cases (standardized incidence rate of 6.3 per 100,000 women) are diagnosed and 140,163 women die of OC annually (estimated in 2008).[1] Mortality is high because women typically present with late-stage disease when the overall 5-year relative survival rate is 45% (reviewed in Ref.2). The most important risk factor is having a family history of the disease (reviewed in Ref.3). Approximately 25% of newly diagnosed cases are due to a hereditary mutation in a single gene: 18% in *BRCA1* or *BRCA2*; 5% in other genes of the Fanconi-Anemia-BRCA DNA repair pathway, including *MRE11, BRIP1, PALB2, RAD51C, CHEK2, NBN, RAD50* and *BARD1*; 1% in genes of DNA mismatch repair pathways (*MLH1, MSH2, MSH6* and *PMS2*) and less than 1% in other genes such as *TP53*.[3–5]

The identification of *RAD51C* mutations in families with breast and ovarian cancer (BC/OC) prompted investigations on the role of another *RAD51* paralog, *RAD51D*, in cancer susceptibility.[6] The protein encoded by *RAD51D* is a member of the RAD51 protein family. The RAD51 protein is a key player in the homologous recombinational repair pathway and its activity appears to be strictly regulated by a number of cofactors including five RAD51 paralogs, namely RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3.[7] RAD51D protein forms a complex with RAD51B, RAD51C and XRCC2 (BCDX2 complex),[8] which is responsible for RAD51 recruitment or stabilization at DNA damage sites.[9] Additionally, it has been described that RAD51D plays a role in telomere protection against attrition and chromosome fusion.[10] Loveday *et al*.[6] identified inactivating mutations in *RAD51D* in eight of the 911 British cases with a familial history of BC/OC (0.88%) and in one of the 1,060 British controls (0.09%). The association was principally found with OC because the higher prevalence of mutations was present in families with more than one case of OC: four mutations in 235 families with two or more cases of OC (1.7%) and three mutations among the 59 families with three or more cases of OC (5.09%).[6] By contrast, no mutations were found in 737 unrelated individuals with only a history of familial BC. The authors estimated an OC relative risk of 6.30 and a BC relative risk of 1.32 for *RAD51D* mutation carriers.[6] Four further studies have explored the role of *RAD51D* mutations in BC/OC families as well as in unselected OC cases.[11–14] These works show that *RAD51D* is an OC predisposition gene, but more studies in familial and sporadic OC series would be of value to further clarify the risks associated to OC and BC.[3]

The aim of our study was to determine the prevalence of germline *RAD51D* mutations in a cohort of Spanish BC and/or OC families previously found to be negative for *BRCA1* and *BRCA2* mutations.

Material and Methods

Patients

The study included a total of 842 unrelated index cases: 713 cases from families with a history of OC and/or BC cases and 129 cases with only a personal history of early-onset BC or OC or concomitant BC and OC. Six centers from Spain have contributed to this study: Hospital Universitari Vall d'Hebron, Barcelona (HUVH) ($N = 281$); Hospital Clínico San Carlos (HCSC), Madrid ($N = 168$); Centro Nacional de Investigaciones Oncológicas (CNIO), Madrid ($N = 165$); Fundación Pública Galega de Medicina Xenómica (FPGMX), Santiago de Compostela ($N = 94$); Hospital de la Santa Creu i Sant Pau (HSCSP), Barcelona ($N = 79$) and Instituto de Biología y Genética Molecular (IBGM), Valladolid ($N = 55$). All index cases were previously screened for point mutations and large rearrangements in *BRCA1* and *BRCA2* genes and no disease-causing mutation was identified.

The studied families were as follows: (*i*) 491 families with one or more OC cases and one or more BC cases (77% of them had at least one case diagnosed before age 50); (*ii*) 171 site-specific female BC families with two or more cases (94% of families with at least one case diagnosed before age 50) and (*iii*) 51 families with two or more OC cases. All the probands analyzed, except 19 healthy cases, were diagnosed with BC (291) or OC (120) or BC + OC (55) or other cancers (6). The study also included patients without family history: 65 BC patients (95% of them diagnosed before age 50), 38 women with OC (82% of them diagnosed before age 50) and 26 with BC and OC. This study was approved by the corresponding institutional ethical committees and informed consent was obtained from all the participants.

Mutation analysis of *RAD51D*

Genomic DNA was extracted from patient peripheral blood samples by standard methods. The screening for germline variation of the coding region and exon–intron boundaries of

the *RAD51D* was performed using different techniques: direct sequencing (HUVH, FPGMX and HSCSP), high-resolution melting (HCSC and IBGM) and denaturing high-performance liquid chromatography (CNIO). All sequence variants were named according to Human Genome Variation Society (HGVS) nomenclature using the NCBI transcript ID NM_002878.3 (or its equivalent sequence from Ensembl transcript ID ENST00000345365) as a reference sequence. The exons were numbered according to the NCBI Reference Sequence: NG_031858.1.

In silico analysis of identified variants

All *RAD51D* genetic variants (coding and noncoding) were analyzed for their potential effect on splicing. For this analysis the variants are reported according to their location, relative to the 5′ (donor) and 3′ (acceptor) consensus sites described by Cartegni *et al.*,[15] that is 11 bases for the donor site (from the three last exonic to the eight first intronic bases) and 14 bases for the acceptor site (from the 12 last intronic to the first two exonic bases). Thus, the variants were classified in three categories: variants occurring at consensus donor or acceptor splice sites, exonic positions outside the consensus sites and intronic positions outside the consensus sites (Supporting Information Table S1). Splicing predictions were performed with Human Splicing Finder, SpliceSiteFinder-like, NNSplice, MaxEntScan and Genscan by using Alamut software v2.1 (Interactive biosoftware). For the nucleotide variants located in consensus sites, if the score estimated by MaxEntScan and SpliceSiteFinder-like was 15 and 5% lower than the wild-type score, respectively, the three remaining prediction algorithms were used (modified from Ref.16). Then, a consensus decrease across all algorithms was considered as indicative of disruption of normal splicing.[6] For variants occurring outside consensus splice sites, the exon skipping, the use of a cryptic site or of a *de novo* splice site, had to be predicted by at least the MaxEntScan and SpliceSiteFinder-like algorithms. We also evaluated the predicted effects of *RAD51D* missense variants on protein using PolyPhen, SIFT and Align GVGD by using Alamut v2.1 and Condel integrated score (http://bg.upf.edu/condel/home).[17]

cDNA analysis

To analyze splice-site mutations total RNA was purified from peripheral blood lymphocytes using the RNA blood mini kits (Qiagen, Hilden, Germany) or TRIzol (Invitrogen, Carlsbad, CA, US). At least two RNAs extracted from normal individuals were used as controls. To generate complementary DNA (cDNA), 1 μg RNA was retrotranscribed with either the superScriptII kit (Invitrogen, Carlsbad, CA, US) or the High-Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, Ca, US). The cDNA was amplified with two sets of primers spanning exons 1–7 (1F-cctctctaggaaggggtaggg and 7R-cagttcctgaagaaccagtca) and exons 5–10 (5F-caggctaaaacccaggatga and 10R-ccaggtcccaatgtctacca). The products were electrophoresed on agarose gels and the resolved cDNA fragments were either directly sequenced or extracted from the gel using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) before bidirectionally sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster city, Ca, US).

Results

We screened the *RAD51D* gene for germline sequence variations in a total of 842 Spanish index cases with either a family history of BC and/or OC or a personal history of BC or OC with early-onset or concurrent BC and OC. We identified 25 different *RAD51D* sequence variants (Table 1). Two variants overtly pathogenic and one likely pathogenic were detected in four families out of the 491 families with BC/OC cases. One of the clear mutations was a deletion affecting a canonical donor splice site leading to loss of exon 7 and a premature stop codon in the *RAD51D* transcript in one family (c.667+2_667+23del, p.Val193Alafs*4) (Fig. 1*a*). A nonsense variant in exon 8 was identified in two unrelated families (c.694C>T, p.Arg232*). Moreover, a substitution likely pathogenic, c.1A>T (p.Met1?), affecting the translation initiation codon was also detected in one family with BC/OC cases. The *in silico* analysis predicted that this variant would cause the activation of a cryptic acceptor site at c.15 (Table 1). No evidence of a new aberrant transcript was obtained in the cDNA analysis of this variant and both wild-type and variant alleles were equally present in the sequence of the cDNA (Fig. 1*b*), suggesting that the c.1A>T does not induce a major alteration in the transcript expression. The cDNA analysis further revealed two alternative transcripts, one containing an out-of-frame skip of exon 3 and the other an in-frame skip of exons 3, 4 and 5 that were present both in the patient and all negative control samples (data not shown). The delta 3 isoform was also found in controls by Osher *et al.*[11] The delta 3, 4 and 5 isoform is described in NCBI database as an alternative transcript NM_133629.2, also known as isoform 4 or TRAD-d3.

The families carrying the *RAD51D* pathogenic variants presented only one OC case (Fig. 2). The splicing variant c.667+2_667+23del was present in the index case, a woman diagnosed of bilateral BC at the ages of 29 and 40, and in two sisters, one diagnosed with BC at the age of 49 and the other with bilateral BC at the age of 43 (Fig. 2). The nonsense variant c.694C>T (p.Arg232*) was present in a woman diagnosed with OC at the age of 44 in one family and in a woman with bilateral OC at the age of 42 in the other family (Fig. 2). In one of these families three healthy sisters and one healthy brother also carried this nonsense variant (Fig. 2). Both the affected and healthy carriers (six subjects) also carried the missense change c.715C>T (p.Arg239Trp) in exon 8 (Table 1). The c.1A>T (p.Met1?) mutation was present in a woman with BC and OC diagnosed at the age of 51 and 64, respectively (Fig. 2).

In addition, we also identified seven *RAD51D* nonsynonymous variants (Table 1). The variants c.494G>A (p.Arg165Gln;

Table 1. Germline *RAD51D* variants identified

Location exon/intron	Nucleotide change	Amino acid change	rs number	*In silico* analyses					MAF (*n*)	MAF 1000 genomes	MAF ESP EA[1]	Previously identified
				ALIGN GVGD	SIFT	POLYPHEN	Condel	Splice site				
Pathogenic mutations												
Exon 1	c.1A>T	p.Met1?		–	–	–		c.15 cryptic acceptor enhanced	0.0006 (842)	–	–	
Intron 7	c.667+2_667 +23del	p.Val193Alafs*4		–	–	–		Disruption of splicing donor site	0.0006 (842)	–	–	
Exon 8	c.694C>T	p.Arg232*		–	–	–		No effect	0.0012 (842)	–	–	Wickramanyake *et al.*[14]: MAF 0.001
Nonsynonymous												
Exon 1	c.26G>C	p.Cys9Ser	rs140825795	C0	Tolerated	Possibly damaging	Deleterious	No effect	0.0006 (842)	–	0.001	Loveday *et al.*[6]: MAF <1%; Wickramanyake *et al.*[14]: MAF 0.001
Exon 5	c.355T>C	p.Cys119Arg	rs201313861	C0	Tolerated	Benign	Neutral	No effect	0.0006 (842)	–	0	Osher *et al.*[11]: MAF 0.003
Exon 6	c.494 G>A	p.Arg165Gln	rs4796033	C0	Tolerated	Benign	Neutral	No effect	0.1087 (842)	0.097	0.143	Loveday *et al.*[6]: MAF > 1%; Pelttari *et al.*[12]: known polymorphism; Osher *et al.*[11]: MAF > 1%; Wickramanyake *et al.*[14]: MAF > 1%; Sellick *et al.*[18]: MAF > 1%
Exon 7	c.629C>T	p.Ala210Val		C65	Deleterious	Probably damaging	Deleterious	No effect	0.0006 (842)	–	0.0001	
Exon 8	c.695G>A	p.Arg232Gln	rs28363283	C0	Tolerated	Benign	Neutral	No effect	0.0029 (842)	0.005	0.0001	Loveday *et al.*[6]: MAF < 1%; Wickramanyake *et al.*[14]: MAF 0.001
Exon 8	c.698A>G	p.Glu233Gly	rs28363284	C0	Tolerated	Possibly damaging	Neutral	No effect	0.0160 (842)	0.006	0.0168	Loveday *et al.*[6]: MAF > 1%; Osher *et al.*[11]:

Table 1. Germline *RAD51D* variants identified (Continued)

Location exon/intron	Nucleotide change	Amino acid change	rs number	*In silico* analyses					MAF (*n*)	MAF 1000 genomes	MAF ESP EA[1]	Previously identified
				ALIGN GVGD	SIFT	POLYPHEN	Condel	Splice site				
												MAF > 1%; Wickramanyake *et al.*[14]: MAF > 1%; Rodríguez-López *et al.*[19]: MAF > 1%; Sellick *et al.*[18]: MAF > 1%; Dowty *et al.*[20]: MAF > 1%; Jara *et al.*[21]: MAF > 1%
Exon 8	c.715C>T[2]	p.Arg239Trp		C0	Deleterious	Possibly damaging	Neutral	No effect	0.0012 (842)	–	–	Wickramanyake *et al.*[14]: MAF 0.001[3]
Synonymous												
Exon 3	c.234C>T	p.Ser78Ser	rs9901455	–	–	–		No effect	0.0736 (842)	0.191	0.082	Loveday *et al.*[6]: MAF > 1%; Osher *et al.*[11]: MAF > 1%; Pelttari *et al.*[12]: known polymorphism; Wickramanyake *et al.*[14]: MAF > 1%; Rodríguez-López *et al.*[19]: MAF > 1%; Sellick *et al.*[18]: MAF > 1%
Exon 9	c.879G>A	p.Ala293Ala		–	–	–		No effect	0.0006 (842)	–	0.0001	Thompson *et al.*[13]: MAF 0.001
Noncoding												
Exon 1 5′UTR	c.-60C>T			–	–	–		No effect	0.0006 (842)	–	–	
Exon 1 5′UTR	c.-111G>A			–	–	–		No effect		–	–	
Intron 1	c.82+128C>T		rs28363258	–	–	–		No effect	0.0088 (455)	0.005	–	Pelttari *et al.*[12]: known polymorphism
Intron 1	c.83-4T>C			–	–	–		No effect	0.0006 (842)	–	–	
Intron 5	c.480+75T>G		rs8067688	–	–	–		c.480+74 *de novo* donor site	0.0029 (338)	0.018	–	

Table 1. Germline *RAD51D* variants identified (Continued)

Location exon/intron	Nucleotide change	Amino acid change	rs number	*In silico* analyses					MAF (*n*)	MAF 1000 genomes	MAF ESP EA[1]	Previously identified
				ALIGN GVGD	SIFT	POLYPHEN	Condel	Splice site				
Intron 5	c.480+158C>A		rs3816754	–	–	–		No effect	0.0479 (94)	0.089	–	
Intron 5	c.480+187T>C		rs936656	–	–	–		No effect	0.3564 (94)	0.478	–	
Intron 5	c.481–205A>G		rs8071313	–	–	–		No effect	0.0159 (94)	0.122	–	
Intron 5	c.481–144T>C		rs8067218	–	–	–		No effect	0.0053 (94)	0.018	–	
Intron 6	c.577–103G>C		rs188753384	–	–	–		No effect	0.0053 (281)	0.001	–	
Intron 8	c.739–103T>C		rs28363289	–	–	–		No effect	0.0202 (173)	0.062	–	
Intron 9	c.903+53C>T		rs45496096	–	–	–		c.903+65 cryptic acceptor enhanced	0.0169 (619)	0.006	–	Pelttari *et al.*[12]: known polymorphism
Intron 9	c.904–11T>A			–	–	–		Disruption of splicing acceptor site	0.0006 (842)	–	0	

[1]ESP EA: minor frequency allele reported by the NHLBI Exome Sequencing Project in European ancestry population.
[2]The carriers of this missense also presented the pathogenic mutation c.694C>T.
[3]Although no clearly stated in the article, the carrier of this missense also presented the pathogenic mutation c.694C>T.

rs4796033) and c.698A>G (p.Glu233Gly; rs28363284) are reported in 1000 genomes and in NHLBI Exome Sequencing Project (ESP) databases as well as in previous reports[18–21] to have allele frequencies near to or greater than 1% (Table 1). Five missense variants, c.26G>C (p.Cys9Ser; rs140825795), c.355T>C (p.Cys119Arg; rs201313861), c.629C>T (p.Ala210Val), c.695G>A (p.Arg232Gln; rs28363283) and c.715C>T (p.Arg239Trp), have been reported with a minor allele frequency (MAF) of 0.0001 to 0.003 in either 1000 genomes and ESP databases as well as in previous studies[11,14,22] (Table 1). Both the c.26G>C and the c.715C>T variants (this last occurring in all the carriers of the deleterious c.694C>T variant) were predicted to be damaging by two of the four programs used to analyze the variant effect on the protein (Table 1). Interestingly, the nonsynonymous variant c.629C>T (p.Ala210Val) located in exon 7 of *RAD51D* was predicted to be deleterious by the four programs used, and is only reported in ESP project with an allele frequency of 0.0001 (Table 1). Similarly, it is also inferred to be potentially damaging based on the degree of conservation of the amino acid among the RAD51 paralogs (Fig. 3). This nonsynonymous variant was identified in a woman affected with OC at the age of 60 and a strong family history of BC/OC cases: one relative with BC and OC diagnosed both at the age of 40, one with bilateral OC diagnosed at the ages of 50 and 55 and one case of endometrial cancer at the age of 58.

We also detected two synonymous changes: c.234C>T (rs9901455) with a MAF >1% and c.879G>A with a MAF <1%. None of these variants were predicted to alter splicing (Table 1 and Supporting Information Table S1). In addition, the mutation screening also revealed 13 noncoding changes: two variants located in the 5′UTR of the gene and 11 intronic sequence alterations. The intronic variants c.480+75T>G, c.903+53C>T and c.904-11T>A were predicted to alter the splicing (Table 1 and Supporting Information Table S1). Unfortunately, no RNA samples were available to study their potential splicing effect.

Discussion

We identified germline *RAD51D* deleterious mutations in 0.82% of the families with at least one OC case and one BC case (4/491). Noteworthy, the four carrier families had seven BC cases. Five were diagnosed before the age 50, including two bilateral BC cases. Previous reports found similar prevalences of *RAD51D* pathogenic variants from 0.57 in Canadian and Belgian BC/OC families[11] to 0.88 in British BC/OC families.[6] Pelttari *et al.*[12] described the presence of a founder *RAD51D* mutation in 2.9% of the Finish patients with a family history of both BC and OC. In contrast, Thompson *et al.*[13] detected no deleterious *RAD51D* changes in 303 Australian BC/OC families. It should be noted that in none of these studies, including ours, the screening techniques used do not allow to detect deletions/duplications affecting complete exons or the entire gene.

We did not identify any deleterious *RAD51D* variant in 171 unrelated individuals from pedigrees with BC cases and

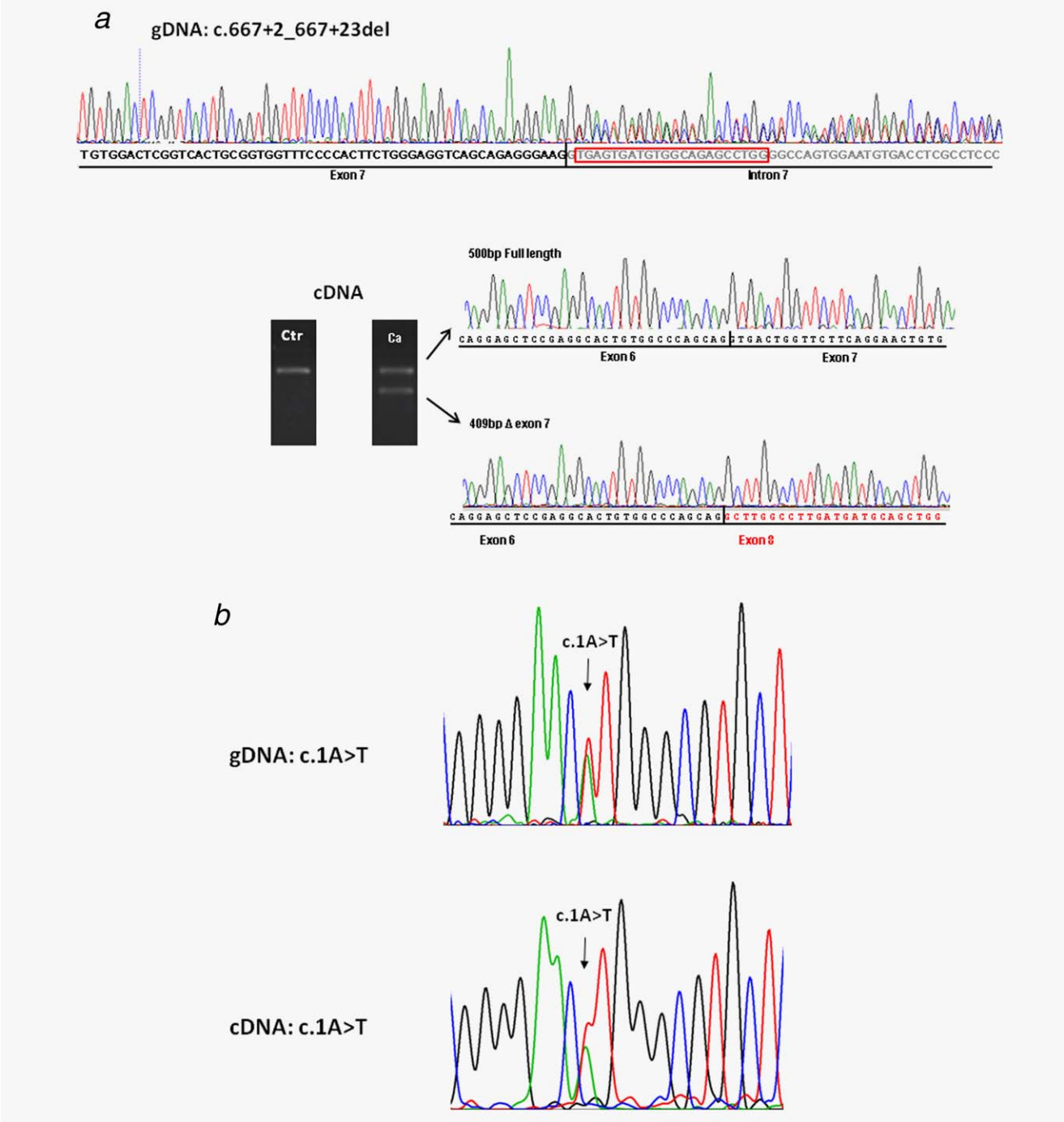

Figure 1. RNA analysis of two *RAD51D* pathogenic mutations. (*a*) Analysis of c.667+2_667+23del; gDNA: electropherogram obtained from genomic DNA showing the 22-bp deletion in heterozygosity in intron 7; cDNA: agarose bands and sequences from cDNA amplification, showing the loss of exon 7 in the mutation carrier (Ca). Ctr: control cDNA. (*b*) Analysis of c.1A>T; sequences obtained from gDNA and cDNA showing the presence of the c.1A>T in heterozygosity. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

no OC cases, which is consistent with other studies that did not find any association between *RAD51D* mutations and BC risk.[6,12–14] Interestingly, no pathogenic variant was detected in our series of 51 pedigrees with only an OC family history. Our data agree with that obtained in previous reports in which a limited number of this type of families was analyzed: eight families[12] and 16 families.[13] Although more studies to assess the implication of *RAD51D* in families with two or more OC cases are required, the results obtained in our larger cohort may suggest that other genes could confer a specific susceptibility to familial OC.

The alteration of the translation initiation codon (c.1A>T, p.Met1?) and the variant leading to a skipping of exon 7 (c.667+2_667+23del, p. Val193Alafs*4) had not been

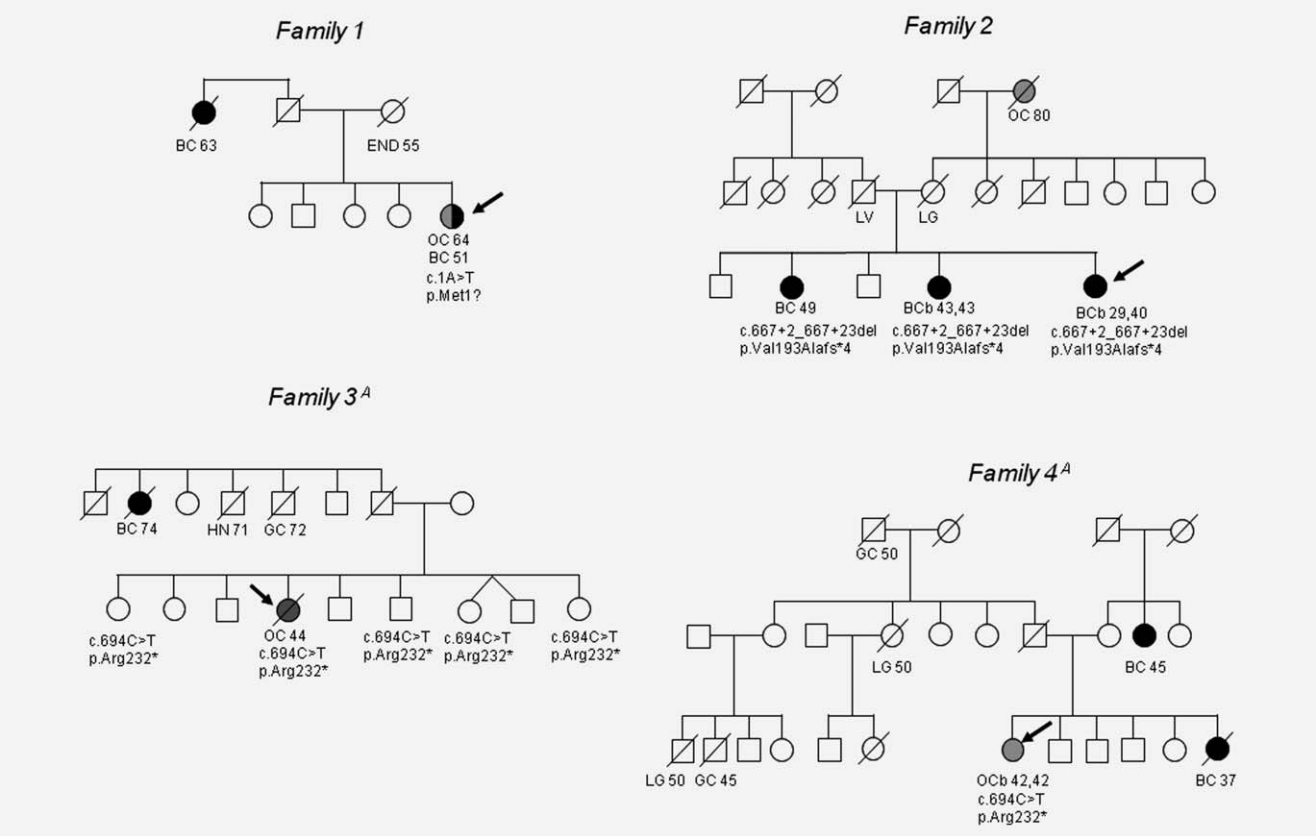

Figure 2. Pedigrees of four families with *RAD51D* pathogenic mutations. The arrow indicates the family proband. Individuals with ovarian cancer are shown as gray circles. Individuals with breast cancer are shown as black circles. Other cancers are shown as unfilled circle. BC: breast cancer; OC: ovarian cancer; BCb: bilateral breast cancer; OCb: bilateral ovarian cancer; HN: head and neck cancer; END: endometrial cancer; GC: gastric cancer; LG: lung cancer; LV: liver cancer. [A]The carriers of the nonsense mutation also presented the missense variant c.715C>T.

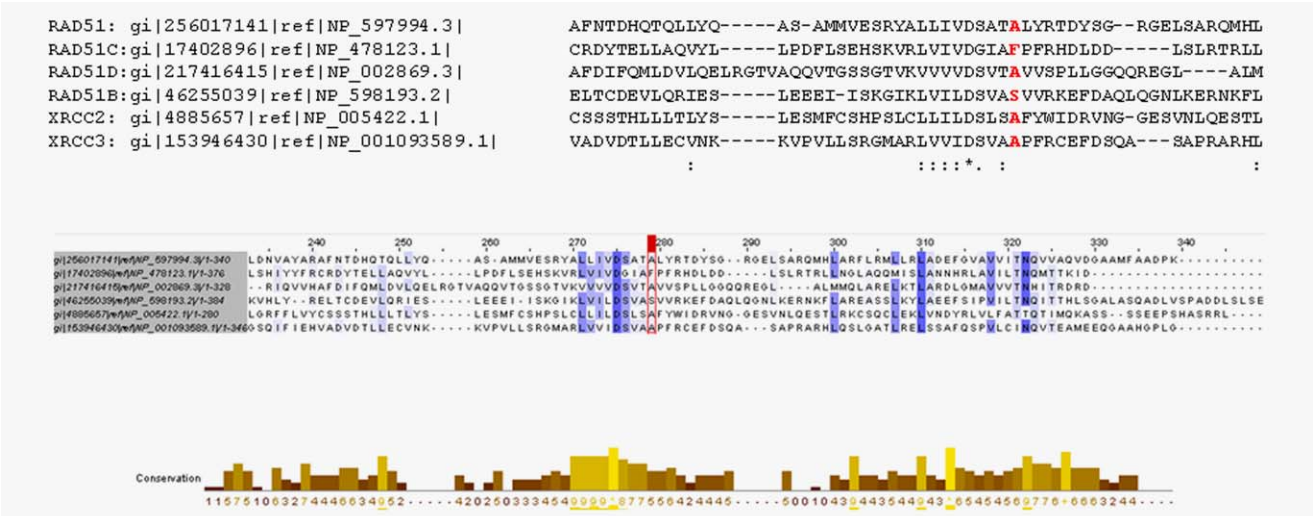

Figure 3. Conservation of site of the *RAD51D* missense variant c.629C>T (p.Ala210Val). RAD51 and its five paralogs were aligned using the Clustal Omega program (multiple sequence alignment program for proteins, http://www.ebi.ac.uk/Tools/services/web/toolform.ebi?tool=clustalo). Fully or partially conserved residues are coloured. An * (asterisk) indicates positions that have a single, fully conserved residue. A: (colon) indicates conservation between groups of strongly similar properties—scoring >0.5 in the Gonnet PAM 250 matrix. A. (period) indicates conservation between groups of weakly similar properties—scoring ≤0.5 in the Gonnet PAM 250 matrix. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

previously described. Given that other single base-pair substitutions located within the ATG translation initiation codon in different genes have been reported to perturb the initiation step of translation, and to reduce translational efficiency *in vivo* (reviewed in Ref.23), we consider the substitution c.1A>T (p.Met1?) pathogenic.

The nonsense variant in exon 9 (c.694C>T, p.Arg232*) has also been identified in a group of American women with OC who had not been selected for age of onset or family history.[14] Interestingly, all carriers of this nonsense alteration in our series (two with OC and four healthy) and the affected carrier found by Wickramanyake *et al.*[14] also carried the nonsynonymous variant in exon 8 c.715C>T. Altogether, these data suggest that both variants are probably in *cis* and the nonsense nucleotide alteration could be a founder mutation.

The role of rare *RAD51D* missense variants in OC risk is not yet clear. Loveday *et al.*[6] did not find overall differences in the frequency of nontruncating *RAD51D* variants between cases and controls. Rare nonsynonymous variants have been further identified among families with BC only[13] and unselected women with OC,[14] but besides *in silico* analysis, were not further assessed for pathogenicity. We identified a nonsynonymous variant, c.629C>T (p.Ala210Val), which is potentially pathogenic based on SIFT, PolyPhen, Align GVGD and Condel predictions, and the amino acid conservation among the RAD51 paralogs (Fig. 3). The change Ala210Val is located in the C-terminal ATPase domain of RAD51D that is conserved among eukaryotic proteins (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=238543) as well as among the five human RAD51 paralogs, and is closely related to the C-terminal domain of RAD51.[24] However, functional assays and cosegregation data with OC in the family are warranted to clarify the impact of this variant.

The studies published to date show that *RAD51D* mutations are more likely to be found in families with two or more OC cases and at least one BC case.[6,11] In contrast, in our study all *RAD51D* mutations were present in families reported with only one OC case and one or more BC cases (Table 2). Our findings suggest that *RAD51D* testing should be offered to affected women with a familiar history of BC/OC regardless of the number of OC cases in the family. Moreover, in our series the splicing variant c.667+2_667+23del was present in three women with BC (two of them had bilateral BC), and only one relative with OC. Loveday *et al.*,[6] Osher *et al.*[11] and Pelttari *et al.*[12] also described *RAD51D* deleterious variants in women affected with BC in BC/OC families. Although the frequency of *RAD51D* mutations in BC families with no OC could be anticipated to be very low,[6] these findings support a higher incidence of breast tumors associated to *RAD51D* mutations, albeit in the context of familial OC.

The apparent excess of BC carriers in these families could be attributed to an ascertainment bias selecting OC cases with a close family history of BC,[6] but also to the existence of other unknown variants that could modify the BC risk associated with *RAD51D* mutations in these families and warrants further investigations.[11,12] The early onset of BC in these families agrees with this latter hypothesis. Combining all data of *RAD51D*-associated BC cases published to date[6,11,12] and our data, a mean age of 44.77 years for BC onset was obtained (Supporting Information Table S2), which is lower than the mean age for sporadic breast tumors (an average age of onset in their 50s for Caucasian women referenced in Haffty *et al.*[25]) and similar to the mean age of BC in *BRCA1* or *BRCA2* mutation carriers reported in diverse populations: 43.6 years (Spanish), 41.6 (British) and 44.1 years (Canadian) for *BRCA1* and 42.8 (Spanish), 45.2 (British) and 47.3 years (Canadians) for *BRCA2*.[26–28] Alternatively, specific mutation risks may be attributed to the type and location of the variants.[12] Considering the mutations described in previously published articles and in this article, we did not observe any association between the BC or OC risk and location of the mutations along *RAD51D* (data not shown).

In conclusion, we identified three pathogenic variants, c.667+2_667+23del, c.1A>T and c.694C>T in four out of the 491 probands (0.82%) from OC and BC families. Our results agree with the association of *RAD51D* mutations with the presence of OC cases in the family. Interestingly, all pathogenic variants were present in families reported with only one OC case, suggesting that *RAD51D* testing should be offered to any affected BC or OC subject irrespective of the number of OC cases in the family. In the c.667+2_667+23del proband's family, both the proband and two relatives carried the mutated allele and were affected with BC (two of them bilaterals). Despite the modest proportion of *RAD51D* deleterious

Table 2. Prevalence of *RAD51D* pathogenic variants reported according to the number of ovarian cases in breast and ovarian cancer families

Reference	N° mutation carriers/n° BC + 1OC families (%)	N° mutation carriers/n° BC + 2OC families (%)	N° mutation carriers/n° BC + ≥3OV families (%)	Total
Loveday *et al.*[6]	1/617 (0.16)	4/235 (1.7)	3/59 (5.1)	8/911 (0.9)
Oscher *et al.*[11]	0/124 (0)	1/43 (2.32)	0/8 (0)	1/175 (0.5)
Pelttari *et al.*[12]	ns	ns	ns	3/105 (2.9)
Thompson *et al.*[13]	0/ns	0/ns	0/ns	0/303 (0)
Our study	4/282 (1.4)	0/189 (0)	0/20 (0)	4/491 (0.8)

Abbreviations: BC: breast cancer; OC: ovarian cancer; ns: not specified.

mutations identified, genetic testing of this gene should be considered into the clinical setting, at least for families with BC and OC. The presence of several BC mutation carriers, albeit in the context of familial OC, suggests an increased risk of this type of cancer, which should be considered when setting the follow-up and prevention measures, and future specific treatments. Additional studies in familial BC/OC series would be of value to provide more complete information on the actual risk of OC, as well as the potential risks of BC and any other cancers.

Acknowledgements

The authors are grateful to the families for their cooperation and to the clinical personnel involved in aspects of recruitment and clinical data collection. SGE and AT are funded by a Miguel Servet contract (CP10/00617) from the Spanish Carlos III Health Institute. The research in HUVH-VHIO (Barcelona) was supported by a Miguel Servet Project grant CP10/00617. HCSC contribution was supported by Fondo de Investigación Sanitaria (FIS) research grants PI 12/00539, an initiative of the Insituto de Salud Carlos III (Spain), partially supported by European Regional Development FEDER Funds. CNIO study was partially supported by Mutua Madrileña Foundation (FMMA), Intrasalud PI12/00070, Acción Cooperativa y Colaborativa Intramural-CIBER2012 and SAF2010-20493. In FPGMX, this study was supported by grants from the Xunta de Galicia (10PXIB 9101297PR) and FMM Foundation given to AV. The research in IBGM is funded by the Cancer Prevention Program of the Regional Government of Castilla y León.

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