

Predominance of pathogenic missense variants in the *RAD51C* gene occurring in breast and ovarian cancer families

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***RAD51C* was defined by Meindl *et al.* in 2010 as a high-risk gene involved in hereditary breast and ovarian cancers. Although this role seems to be clear, nowadays there is controversy about the indication of including the gene in routine clinical genetic testing, due to the lower prevalence or the absence of mutations found in subsequent studies. Here, we present the results of a comprehensive mutational screening of the *RAD51C* gene in a large series of 785 Spanish breast and/or ovarian cancer families, which, in contrast to the various subsequent studies published to date, includes the functional characterization of suspicious missense variants as reported in the initial study. We have detected 1.3% mutations of *RAD51C* in breast and ovarian cancer families, while mutations in breast cancer only families seem to be very rare. More than half of the deleterious variants detected were of missense type, which highlights their significance in the gene, and suggest that *RAD51C* mutations may have been so far partially disregarded and their prevalence underestimated due to the lack of functional complementation assays. Our results provide new evidences, suggesting that the genetic testing of *RAD51C* should be considered for inclusion into the clinical setting, at least for breast and ovarian cancer families, and encourage re-evaluating its role incorporating functional assays.**

INTRODUCTION

Only around 25% of the inherited susceptibility to breast cancer is explained by germ-line mutations in the high-penetrance susceptibility genes *BRCA1* and *BRCA2* (1–4). Apart from those, mutations in genes such as *TP53* and *PTEN* have been linked to a high risk for breast cancer within well-defined cancer syndromes (5,6). Germ-line mutations in other genes, such as *CHEK2* (7), *ATM* (8) and more recently the Fanconi anemia (FA) genes *BRIP1/FANCF* and *PALB2/FANCD1* (9,10), have been found to confer small but

distinct risks for breast and other cancers. The clinical significance of those risk alleles is under debate as they account altogether for less than 5% of hereditary cases of breast cancer, in some instances are restricted to distinct ethnic groups or populations, and the risk they convey generally ranges between no more than 2- and 4-fold, all of which makes them faint targets for genetic assessment. Only the most frequent mutations in the *CHEK2* gene, 1100delC and EX9_10del (also designated as del5395), have been associated with clinically meaningful risks in a familial context, and their

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integration into early recognition programs has been recently indicated (11).

An important finding was published by Meindl *et al.* (12), showing that heterozygous mutations in the FA gene *RAD51C* (13) were able to confer an increased risk for breast and ovarian cancers. What attracted particular interest is that mutations in *RAD51C* seem to be highly penetrant in breast and ovarian cancer families, suggesting the possibility of a third high-risk gene predisposing to these types of cancer.

After this role of *RAD51C* was recognized, several studies have been performed in different populations trying to confirm that finding. At least eight studies have been conducted in series each including 100 or more patients of various origins, showing divergent results. While in four of these studies, undertaken in families from the USA (14,15), Canada (enriched in Jewish and French-Canadian ethnic origin) (16) and China (17), high-penetrance *RAD51C* mutations could not be detected, in three most recent Spanish, Finnish and Swedish series, truly truncating mutations were identified in the gene (18–20). In the Finnish study, two putative *RAD51C* founder mutations were observed to confer a moderate-to-high risk for ovarian cancer. Finally, the most extensive study published to date reported 0.6% truncating mutations in a series of 1388 Australian breast and/or ovarian cancer families (21). These latter results confirmed the role of *RAD51C* as a susceptibility gene in breast/ovarian and ovarian cancer only families, at least for specific populations, but on the other hand suggest that the prevalence of these mutations may be lower than stated in the initial report (10).

Notably, in all of the mentioned studies, a considerable number of unique *RAD51C* missense variants were observed in the gene, which were not further assessed for pathogenicity and discounted. Given the fact that in the first report by Meindl *et al.* (12), one third of the deleterious *RAD51C* sequence alterations were missense mutations, we cannot rule out the possibility that *RAD51C* mutations in these studies may have been partially disregarded and their prevalence underestimated. As we might be potentially facing with *RAD51C*, a high-risk breast and ovarian cancer susceptibility gene, there is an urgent need to clarify its role in a more comprehensive manner. Here, we have investigated a large series of 785 Spanish breast/ovarian cancer families for all types of mutations in *RAD51C*. We have detected four novel mutations and one previously reported (19), all of them in non-*BRCA1/BRCA2* families. Three of the novel mutations were missense variants, whose DNA repair-impairing character was demonstrated by functional assay. Our results convey and extend the significance of *RAD51C* mutations in breast/ovarian cancer families and highlight the necessity of assessing the pathogenicity of the missense variants occurring relatively often in this gene.

RESULTS

RAD51C gene variants

The whole coding sequence and exon–intron boundaries of the *RAD51C* gene were analyzed in the index cases of 485 breast only and 300 breast and/or ovarian cancer non-*BRCA1/2* Spanish families. A total of 17 variants were

identified; those found in the Hospital Clínico San Carlos (HCSC) series have previously been reported by Romero *et al.* (19), as indicated in Table 1. Seven of the 17 variants were considered neutral either because they had a reported frequency in CEU (<http://www.ncbi.nlm.nih.gov/SNP/>) or were located at intron positions where they were not predicted to affect the splicing process. Ten of the variants were unique and classified as either clearly deleterious or of unknown clinical significance (Table 1). Prior to functional assays, two truly pathogenic variants were sorted out, c.414G>C; p.Leu138Phe and c.774delT; p.Arg258fs. These were directly considered deleterious mutations, since the former had previously been demonstrated to affect protein functions (12,22) and the latter resulted in protein truncation and had previously been reported as mutation by Romero *et al.* (19). Of the other eight unique non-synonymous changes, three, c.404G>A, p.Cys135Tyr; c.428A>G, p.Gln143Arg; and c.656T>C, p.Leu219Ser, were inferred to be potentially damaging based on predictions made by SIFT (23) and PolyPhen (24) and the degree of conservation among the RAD51 paralogs (Fig. 1). p.Gln143Arg had previously been reported as an unknown significance variant by Romero *et al.* (19) but no functional assays had been performed to assess its pathogenicity. These are presented herein.

In terms of the type of mutation, c.404G>A affects the last nucleotide position of exon 2 and is predicted to destroy the splice donor site of intron 2 by three splice site prediction algorithms integrated in the software Alamut 2.0 (SpliceSiteFinder-like, MaxEntScan and NNSPLICE). This variant might be a splice rather than a missense mutation. As a second blood sample of the corresponding patient was not available and studies at the RNA level were not feasible, splicing studies could not be performed. Thus, we set forth to characterize the above three novel *RAD51C* variants as missense mutations, including c.404G>A (p.Cys135Tyr) in case it might not be a fully acting splice site mutation.

Characterization of missense mutations

Proficient RAD51 foci formation functionally assesses the potential of cells to exert homology-directed DNA repair (25). As shown by Vaz *et al.* (13), SH2038-F primary fibroblasts deficient of RAD51 foci formation ($2.8 \pm 2.2\%$ foci-positive cells compared with $31.6 \pm 6.9\%$ in normal controls) could be restored to normal by retroviral transduction with *RAD51C* wild-type cDNA ($25.9 \pm 4.0\%$ foci-positive cells). Thus, genetic complementation resulted in a functional correction. A similar approach, using site-mutated *RAD51C* cDNA, was taken by Meindl *et al.* (12) to assay the mutational character of *RAD51C* missense variants observed in breast/ovarian cancer families. Here, we followed a slightly modified procedure using large-T antigen-transformed SH2038-F fibroblasts, for which the range between uncorrected and corrected RAD51 foci rates is wider than for primary fibroblasts, and we employed transfection rather than transduction techniques which proved to be less time-consuming. The expression vector pCMV-Tag3bRAD51C (Supplementary Material, Fig. S1) was able to complement *RAD51C*-deficient cells, whereas the matched vector, containing *RAD51B* cDNA, was not (Table 2). Three categories of cells resulted from

Table 1. Genetic variants identified in *RAD51C* during the screening on 785 Spanish breast and/or ovarian cancer families

Exon	Nucleotide change ^a	Protein change	Previously described	<i>n</i> ^b	MAF reported in CEU	Family phenotype ^c	SIFT	PolyPhen	Clinically important ^d
5'UTR	c.-118G>A	—	rs16943176	—	0.205	—	—	—	No
5'UTR	c.-26C>T	—	rs12946397	—	0.2	—	—	—	No
1	c.89C>A	p.Ala30Glu	Novel ^e	1	—	BC	Tolerated	PbD	Unknown
1	c.106G>A	p.Glu36Lys	Novel ^e	1	—	BC	Tolerated	Benign	Unknown
1	c.134A>G	p.Glu45Gly	Novel ^e	1	—	BC	Tolerated	Benign	Unknown
IVS 1	c.146-67dup	—	Novel ^e	1	—	BC	—	—	No
2	c.376G>A	p.Ala126Thr	rs61758784	—	0.003	—	Tolerated	Benign	No
2	c.404G>A	p.Cys135Tyr	Novel	1	—	BC/OC	Deleterious	PbD	Yes
3	c.414G>C	p.Leu138Phe	Yes^f	1	—	BC/OC	Deleterious	PsD	Yes
3	c.428A>G	p.Gln143Arg	Novel^e	1	—	BC	Deleterious	PsD	Yes
IVS 3	c.572-17G>T	—	Yes ^g	—	—	—	—	—	No
4	c.586T>C	p.Leu196Leu	Novel ^e	1	—	BC	—	—	Unknown
4	c.656T>C	p.Leu219Ser	Novel	1^h	—	BC/OC	Deleterious	PsD	Yes
5	c.774delT	p.Arg258fs	Novel^e	1	—	BC/OC	—	—	Yes
6	c.859T>C	p.Thr287Ala	rs28363317	—	0.022	—	Tolerated	PbD	No
6	c.869T>C	p.Ile290Thr	Novel ^e	1	—	BC	Tolerated	Benign	Unknown
IVS 6	c.904 + 34T>C	—	rs28363318	—	0.292	—	—	—	No

PbD, probably damaging; PsD, possibly damaging; BC, breast cancer only family; BC/OC, breast and ovarian cancer family.

^aMutation nomenclature listed uses GenBank reference sequences NM_058216.1 with numbering starting at the A of the first ATG, following the journal guidelines (www.hgvs.org/mutnomen).

^bNumber of index cases in which the variant was found in the present series. Indicated for changes not reported in SNP data base only.

^cIndicated for novel variants only.

^dBased on the functional studies detailed in the text. Variants that were classified as deleterious are in bold. Variants that had a reported frequency in CEU (<http://www.ncbi.nlm.nih.gov/SNP/>) or were located in introns in positions that were not predicted to affect the splicing process were considered as neutral. All the intronic variants that were considered as neutral were predicted not to affect the splicing process by the four splice site prediction algorithms integrated in the software Alamut 2.0 (SpliceSiteFinder-like, MaxEntScan, NNSPLICE and Human Splicing Finder).

^eVariants found in the HCSC series reported in a single case but previously published in Romero *et al.* (19).

^fReported as deleterious mutations in the original study by Meindl *et al.* (12).

^gFound in both CNIO and HCSC and reported in Pang *et al.* (17) and Romero *et al.* (19).

^hIn this case, a specific screening by denaturing high performance liquid chromatography (DHPLC) was performed in a series of 550 controls of the general population that were all found to be negative, confirming the pathogenicity of the variant.

our expression assay: first, native normal control cells and *RAD51C*-deficient cells complemented with wild-type *RAD51C* had >50% nuclei with $\gg 10$ *RAD51* foci per nucleus (Fig. 2A). Second, an intermediate class of *RAD51C*-deficient cells expressing *RAD51C* variants showed <50% *RAD51* foci-positive nuclei with reduced numbers of foci per nucleus (Fig. 2B). Third, uncorrected or mock (*RAD51B*)-transfected *RAD51C*-deficient cells had a minimum rate of *RAD51* foci-positive nuclei and greatly reduced numbers of *RAD51* foci per nucleus (Fig. 2C). Quantitative evaluation of the results with the three novel missense variants, c.404G>A (p.Cys135Tyr), c.428A>G (p.Gln143Arg) or c.656T>C (p.Leu219Ser), expressed in *RAD51C*-deficient SH2038-F fibroblasts, is shown in Figure 3. None of these variants were able to restore *RAD51* foci formation close to normal controls and hence complement the cells. All rates of *RAD51* foci-positive cells that the variants were able to produce were significantly less than the proportion of cells corrected by wild-type *RAD51C* cDNA. Thus, they were all considered deleterious sequence variants and authentic mutations. While p.Cys135Tyr and p.Gln143Arg were still able to engender intermediate rates with less than half of *RAD51* foci-positive cells compared with wild-type cDNA, c.656T>C (p.Leu219Ser) yielded virtually negative rates. Numerical (number of foci-positive cells) and morphological (number of foci in nuclei of positive cells) assessment of *RAD51* foci formation with the missense variants and positive

and negative controls are summarized in Table 2. These results confirm each other and provide combined evidence that the three missense variants compromise the DNA repair capacity of *RAD51C*, consistent with the definition of missense mutations.

Segregation analysis

Extended pedigrees of the patients harboring the five deleterious variants in *RAD51C* are shown in Figure 4 and detailed below.

p.Cys135Tyr (Fig. 4A) was detected in a woman affected with breast at 64 and bilateral ovarian cancer at 73 years of age, who had a sister affected with ovarian cancer at 54 and a maternal cousin with breast cancer at 47.

p.Leu138Phe (Fig. 4B) was observed in a patient diagnosed with bilateral ovarian cancer at 62 years who had two sisters affected with breast cancer at 37 and bilateral breast cancer at 64 and 72. In this case, we were able to confirm the segregation of the mutation with the disease in the sister affected with bilateral breast cancer. p.Leu138Phe had been reported by Meindl *et al.* (12) in a German breast and ovarian cancer pedigree, but the Spanish patient did not report German ancestors. Pathogenicity of this variant has recently been confirmed by functional assays (22).

p.Leu219Ser (Fig. 4C) was found in a breast cancer patient diagnosed at 44 who had a sister and her mother affected with

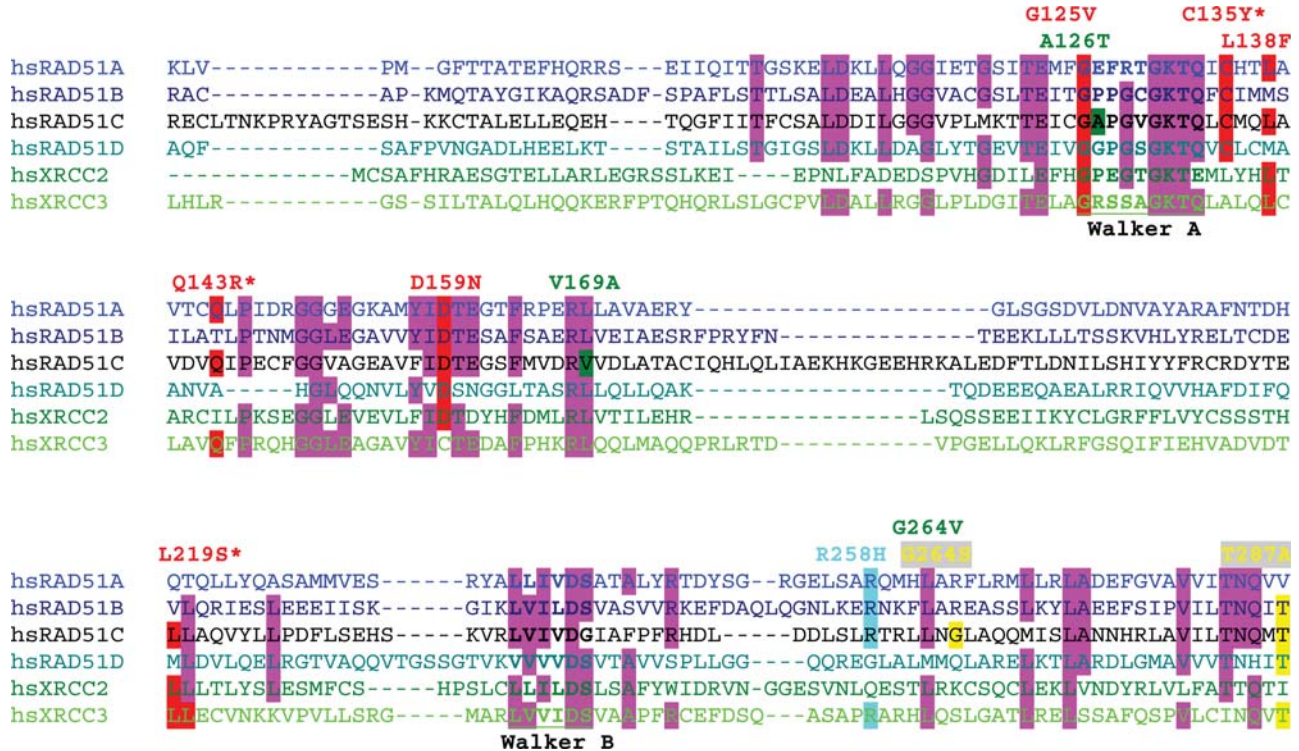


Figure 1. Conservation of sites of *RAD51C* missense mutations. *RAD51* and its five paralogs have been aligned. Fully or partially conserved residues are colored; published (12,13) or novel (asterisk) missense mutations are indicated on top.

Table 2. Abundance of RAD51 foci in RAD51C-mutant, RAD51 foci-deficient fibroblasts expressing RAD51C missense variants

Designation	Frequency of RAD51C-pos. cells (mean ± SD) ^a	Number and size of RAD51C foci per pos. nucleus (morphologic assessment)
RAD51C wt	64.24 ± 4.98	+++
C135Y	29.52 ± 4.26	+/-
Q143R	26.06 ± 2.30	+
L219S	10.23 ± 1.66	—
RAD51B	9.62 ± 4.32	—

+++ , >50% nuclei with >>10 RAD51 foci per nucleus; + and +/- , <50% RAD51 foci-positive nuclei with reduced numbers of foci per nucleus; —, the minimum rate of RAD51 foci-positive nuclei with greatly reduced numbers of RAD51 foci per nucleus.

^aBased on three to four independent experiments.

breast cancer at 60 and 63 years of age, respectively, and another sister affected with breast and ovarian cancer at 40 and 50 years. The sister affected with breast cancer at 60 was also carrying the variant.

c.774delT (Fig. 4D) has previously been published by Romero *et al.* (19) and was identified in a woman affected with breast cancer at 36 years of age. We were able to confirm that the variant was inherited from her mother, who had a sister affected with ovarian cancer at 58. Interestingly, the patients' mother was Swedish and this mutation has recently

been observed in a Swedish breast and ovarian cancer family, suggesting that it might be a recurrent or a founder mutation in that population (20).

p.Gln143Arg has also been reported by Romero *et al.* (19) and occurred in a breast cancer only family (Fig. 4E). The index case developed bilateral breast cancer at 56 years of age. She had a sister affected with breast cancer at 36 years of age. The patient's mother had been diagnosed with breast cancer at 74 years but it was not possible to assess her carrier status. There was not any information available about the patient's paternal branch.

Carrier manifestations, epidemiology and features of the *RAD51C*-associated tumors

We were able to test three other women in addition to the index patients (eight women in total) for the presence of the corresponding *RAD51C* familial mutation, and they were all mutation carriers. Considering the eight carriers, all except one were affected with cancer, three of them with breast cancer, two with bilateral breast cancer, one with bilateral ovarian cancer and one with breast and bilateral ovarian cancer. The only unaffected carrier was the mother of the index case in the family carrying the c.774delT mutation (Fig. 4D) who was cancer-free at 70 years of age.

The mean age of onset at the first diagnosis of the carrier patients was 46.28 years (range: 36–64) for breast cancer, which is lower than the mean age for their counterpart sporadic tumors, but higher than in *BRCA1* and *BRCA2* mutation carriers, being 43.6 and 42.8 years, respectively, in Spanish

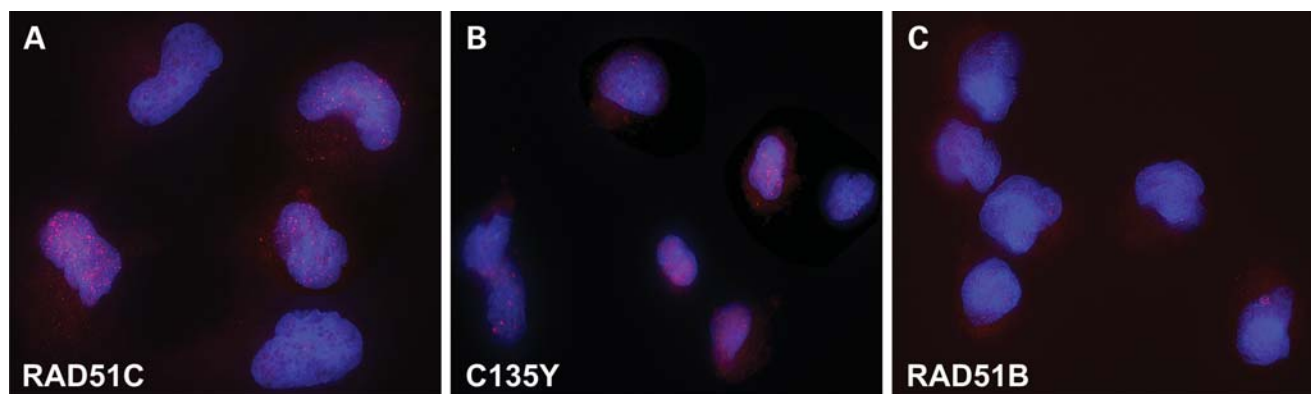


Figure 2. Different degrees of complementation of large T antigen-transformed *RAD51C*-mutant SH2038-F fibroblasts. (A) Cells fully complemented by wild-type *RAD51C* cDNA show >50% (three of five) nuclei with $\gg 10$ RAD51 foci per nucleus. (B) Cells partially complemented by *RAD51C* missense (C135Y) cDNA display <50% (three of seven) RAD51 foci-positive nuclei with reduced numbers of foci per nucleus compared with (A). (C) Cells mock-complemented by *RAD51B* cDNA have a low rate of RAD51 foci. The single positive of six nuclei reveals small foci of a reduced number compared with (A).

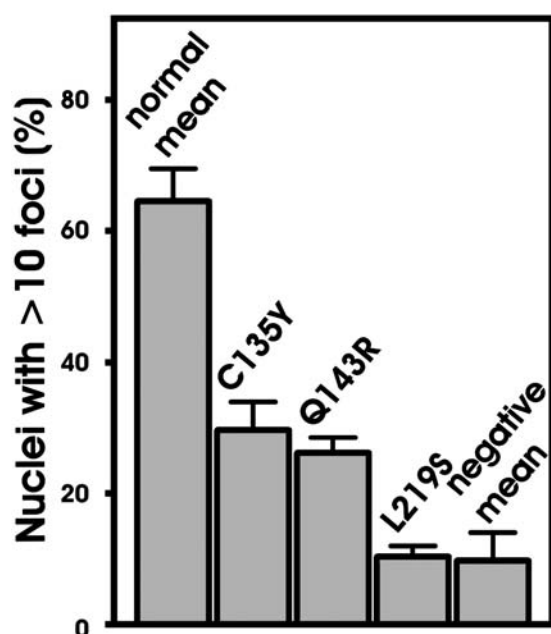


Figure 3. Diminished capability of *RAD51C* missense variants to restore RAD51 foci formation when expressed in large T antigen-transformed *RAD51C*-mutant SH2038-F fibroblasts. The bar graph showing normal (*RAD51C* wild-type complemented) and negative (*RAD51B* mock-complemented) rates of nuclei with >10 RAD51 foci. The proportions of RAD51 foci-positive nuclei for the missense variants C135Y and Q143R are intermediate. They display distinctly impaired rates, while L219S reveals virtually negative values. The means and standard deviations of at least three independent experiments are denoted.

population (1). In terms of ovarian cancer, the mean age of onset is 52 and 55 years for *BRCA1* and *BRCA2* mutation carriers in Spanish population, respectively, and 62 years for sporadic cases (I. Muñoz *et al.*, submitted). The present study includes only two patients affected with ovarian cancer who were confirmed *RAD51C* mutation carriers. They were diagnosed at 73 and 62 years of age, respectively, and in both cases, the affection was bilateral.

Clinico-pathological characteristics of the *RAD51C*-associated tumors are shown in Table 3. These tumors did not share any distinctive feature, except that all breast cancer cases were ductal infiltrating and negative for HER2. The two ovarian cancer cases were serous and high grade.

DISCUSSION

Since the identification of *BRCA1* and *BRCA2* more than 15 years ago, no other high-penetrance susceptibility genes had been found to be specifically involved in the hereditary breast and ovarian cancer syndrome. This appeared to change when Meindl *et al.* showed in 2010 (12) by a combination of genetic and functional assays that highly penetrant mutations in the *RAD51C* gene occurred in 1.3% of breast and ovarian cancer families. This exciting finding has motivated the initiation of various subsequent studies during the last months, trying to confirm the observation in different populations. However, some have failed to detect clearly deleterious mutations in *RAD51C* (14–17), and others have reported highly penetrant mutations in a lower percentage than the described in the initial study (18–21).

Here, we present the results of a comprehensive mutational screening of the *RAD51C* gene in a large series of 785 Spanish breast and/or ovarian cancer families. In contrast to the various studies published to date, suspicious missense variants were characterized for pathogenicity using the same functional assays performed in the study by Meindl *et al.* (12), with slight technical modifications. Altogether five deleterious sequence variants were detected. Notably, three of them, p.Leu138Phe, p.Gln143Arg and p.Leu219Ser, were missense mutations, while one, c.774delT, was a truncating mutation, and p.Cys135Tyr does not only cause a functionally impaired protein, but is also predicted to produce a transcript that is prone to aberrant splicing. Consistent with the observation of Meindl *et al.* (12), four of the mutations were found in breast and ovarian cancer families. According to this figure, the percentage of Spanish breast and ovarian cancer families associated with mutations in *RAD51C* is 1.3% (4 of 300), which is the same as that found in the German study (12). If

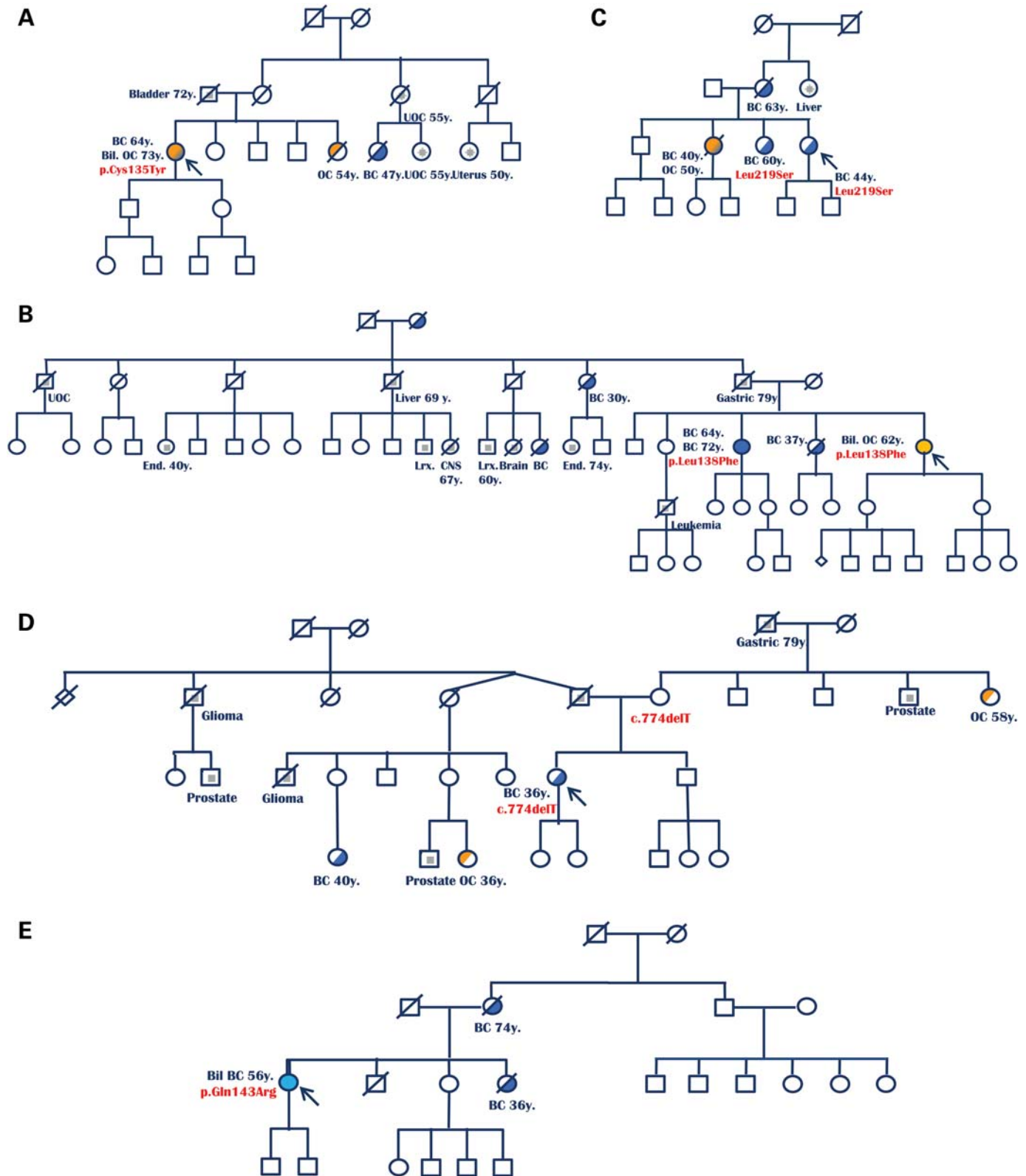


Figure 4. (A–E) Families harboring the five deleterious variants in *RAD51C*. Individuals with breast cancer (BC) are shown as half-filled blue and those with ovarian cancer (OC) as half-filled orange circles. Filled circles represent bilateral tumors, either the breast or the ovarian. Other cancers diagnosed in the family are shown with a grey circle inside with the affected organs indicated (UOC, unknown origin cancer; End, endometrial cancer; BN, brain; Lrx, larynx; CNS, central nervous system). The age at diagnosis is also shown. Index cases are highlighted by an arrow. Mutation carriers are denoted with the name of the mutation in red.

Table 3. Tumor features of the seven *RAD51C*-affected mutation carriers

Individual	Mutation	Type of tumor	Age of onset	Tumor histology	Grade	Stage	ER status	PR status	HER2 status
1	p.Cys135Tyr	Breast	64	IDC	2	—	Positive	Positive	—
		Bilateral ovarian ^a	73	Serous	3	—	Positive	Negative	Negative
2	p.Leu138Phe	Bilateral ovarian ^a	62	Serous	3	IIB	Positive	Positive	Negative
3	p.Leu138Phe	Bilateral breast ^a	64	IDC	3	—	Negative	Negative	Negative
4	p.Gln143Arg	Right breast	56	IDC	2	IIB	Positive	Positive	Negative
		Left breast	56	IDC	2	IIA	Positive	Positive	Negative
5	p.Leu219Ser	Breast	44	IDC	—	—	—	—	—
6	p.Leu219Ser	Breast	60	IDC	3	—	Positive	Positive	—
7	p.Arg258fs	Breast	36	IDC	1	—	Positive	Positive	Negative

IDC, infiltrating ductal carcinoma.

^aAll data shown in the table correspond to the tumor diagnosed first.

functional studies for missense mutations had not been done and this type of mutation had been disregarded, we would have reported a percentage of 0.6% at the most to be linked to *RAD51C*, based on the presence of the truncating mutation c.774delT and the prediction of p.Cys135Tyr as a splice-site mutation. Therefore, our results confirm that missense mutations are definitely relevant in the case of *RAD51C* and that the prevalence of mutations reported so far has been underestimated by the lack of functional assays.

Clearly, the proportion of missense mutations varies greatly from gene to gene. There are genes that are largely tolerable to missense changes so that virtually no missense deleterious mutations occur therein. An example for absent missense mutations would be the gene encoding FANCF, which has been termed 'a flexible adapter protein' because of this characteristic (26). Conversely, there are genes such as *PYGL*, in which a majority of affected alleles (68–80%) reveal missense mutations, resulting in glycogen storage disease type VI, McArdle disease, inherited as an autosomal recessive trait. This could also be the case of *RAD51C* and other genes connected to DNA repair (27).

The characterization of the four *RAD51C* missense mutations reported in this study is based on three consecutive approaches: (i) the demonstration of evolutionary conservation of the original amino acid among RAD51 and its five paralogs (Fig. 1), (ii) the usage of prediction programs for the pathogenicity of a substitution (23,24) and, most importantly, (iii) the expression of the variant in functional complementation assay (Figs 2 and 3). With the conservation of an amino acid in at least three members of RAD51 or paralogs and the prediction of pathogenicity of a substitution via the two *in silico* programs SIFT and PolyPhen, the functional significance of missense variants could be confirmed by the functional assay in all cases.

As also recognized in other studies, *RAD51C* mutations seem to be more or less restricted to breast and ovarian cancer families (Fig. 4A–D). Only one of the five index cases, in which an *RAD51C* mutation was detected (p.Gln143Arg), did not have ovarian cancer herself nor had any relative (Fig. 4E) (19). However, in contrast to Peltari *et al.* (18), we did not observe a preponderance of ovarian cancer compared with breast cancer in our families and it seems therefore not justified to reduce *RAD51C* to a predisposing gene for ovarian cancer. Notably, Thompson *et al.* (21) recently reported several missense variants in the

RAD51C gene, which were detected in breast cancer only families and which were *in silico* predicted to affect protein function. Currently, we are testing whether their prognosticated pathogenicity can be substantiated by the assay employed within this study.

It is noteworthy that four of the seven affected women who were shown to be *RAD51C* mutation carriers developed bilateral tumors, either breast or ovarian, including one patient diagnosed with breast cancer at 64 years of age and bilateral ovarian cancer at 74 (Fig. 4A). Regarding all pedigrees, there was only one family, (c.774delT, Fig. 4D), which did not show any member with a bilateral tumor. The association of *RAD51C* mutations with the development of two or more tumors has previously been suggested in a large study performed in Australian population, where two of three truncating mutations identified in the *RAD51C* gene were observed in women diagnosed with both breast and ovarian cancers (21).

In terms of the age of onset, the mean for breast cancer in *RAD51C* mutation carriers was 46.28 years, which is intermediate between the age of onset in *BRCA1* and *BRCA2* mutation carriers and their sporadic counterparts in Spanish population (1). This is consistent with the observation by Meindl *et al.* (12), who reported very similar figures in German population. Also consistent with the reports by Meindl *et al.* (12) and Peltari *et al.* (18) are the facts that all breast tumors in our study were ductal and in general poorly differentiated, with no consistency for the hormonal status. The four breast tumors, for which we had information on the HER2 status, had a negative score, which again coincides with the initial study by Meindl *et al.* (12), where six breast tumors, in which the HER2 status was assessed, were negative for this marker. However, the investigation of a larger number of *RAD51C* mutation-associated breast tumors are necessary to confirm whether negativity for HER2 could be a typical feature of *RAD51C*-associated tumors as it has been described for *BRCA1*- and *BRCA2*-related breast cancers (28).

In summary, we have detected 1.3% mutations of *RAD51C* in a large series of Spanish breast and/or ovarian cancer families (4 of 300), while mutations in breast cancer only families seem to be very rare (1 of 438). More than half of the mutations detected were of missense type, which highlights the significance of such mutations in the *RAD51C* gene, only recognized by functional complementation assays. Mutations in *RAD51C* might be associated with the occurrence of

bilateral tumors or primary cancers of different types, but further studies are required to confirm our observations. These are new and enough evidences, suggesting that the genetic testing of *RAD51C* should be included into the clinical setting, at least for breast and ovarian cancer families, and a re-evaluation of its role incorporating functional assays is needed.

MATERIALS AND METHODS

Non-BRCA1/2 families

Index cases from 785 Spanish breast and/or ovarian cancer families recruited through the Spanish National Cancer Centre (CNIO) ($n = 293$) and HCSC ($n = 492$) were included in the present study, some of them reported previously (1,2,19). Four hundred and eighty-five families were breast cancer only, showing either one breast cancer at age ≤ 35 with no other family history or at least two first-degree relatives diagnosed with breast cancer at least one of them at age 50 or younger. Three hundred families fulfilled the same criteria but included at least one ovarian cancer case. DNA isolated from peripheral blood of the 785 cases, had been fully screened for mutations in *BRCA1* and *BRCA2* by a combination of denaturing high performance liquid chromatography (DHPLC), high resolution melting (HRM) and direct sequencing and was judged to be negative (1,29,30). This study was approved by the local Medical Ethical Committee. Informed consent was obtained from each participant. Personal and cancer family histories were obtained from the proband and participating relatives. Cancer diagnoses and deaths were confirmed by reviewing medical records, pathology reports or death certificates.

Controls

DNA samples from 550 women collected from the Spanish College of Lawyers aged between 25 and 65 and without personal or familial antecedents of any type of cancer were analyzed to establish the frequency of some of the *RAD51C* variants in the general population.

Molecular analysis of the *RAD51C* gene

DNA was isolated from blood leukocytes of the 785 index cases and 500 controls using MagNA Pure LC Total Nucleic Acid Extraction. DNAs from HCSC were screened by HRM using primers and methods described previously (19). For the screening performed at CNIO, nine primer pairs were designed to amplify the nine coding exons, exon-intron boundaries and part of the 5' and 3' untranslated regions (UTR) of the *RAD51C* gene (NM_058216.1) using the program Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (primer sequences available upon request). The polymerase chain reaction products were analyzed by DHPLC on the WAVE HT system (Transgenomic, Omaha, NE, USA) using an acetonitrile gradient and were scrutinized for aberrant profiles with the Navigator™ software (Transgenomic). Samples representative of each abnormal DHPLC pattern were bidirectionally sequenced using the Big Dye Terminator Cycle sequencing kit and a

3730 automated sequencer (ABI Perkin Elmer). All variants not previously described were confirmed by re-sequencing of a fresh aliquot from stock DNA.

Functional characterization of *RAD51C* missense variants

Human full-length *RAD51C* cDNA (Ensembl Transcript ID ENST00000337432, RAD51C-001) was directionally *EcoRI* > *XhoI* cloned into the mammalian expression vector pCMV-Tag3b (Stratagene) to result in the complementation vector pCMV-Tag3bRAD51C (Supplementary Material, Fig. S1). The *RAD51C* insert contained the synonymous single nucleotide polymorphism, c.216A>G (p.K72K). As a control, *RAD51B* cDNA was cloned in the same way. Putatively deleterious sequence variants observed in breast/ovarian cancer families were introduced into the wild-type *RAD51C* sequence using mismatch primers and PfuUltra II Dna polymerase (Agilent). All site-mutated inserts were verified by sequence analysis. Following plasmid preparation, the different varieties of the *RAD51C* vector were transfected into the large T antigen-transformed *RAD51C*-mutant cell line SH2038-F (human fibroblasts derived from a patient with FA, carrying a homozygous mutation of *RAD51C*, c.773G>A; p.R258H) (13). For that purpose, the fibroblasts were seeded into slide flasks (Nunc) at a confluency rate of 50–80%. The growth medium was changed to 1.5 ml of minimum essential medium alpha (α -MEM) containing 5% fetal calf serum (FCS) 30 min before transfection. At the same time, we prepared a transfection mixture consisting of 300 μ l of α -MEM with 5% FCS, 6 μ l of plasmid DNA (100 ng/ μ l) and 6 μ l of X-tremeGENE HP DNA Transfection Reagent (Roche). Following incubation at retro transcription, the mixture was added dropwise to each flask while pivoting them gently. G418 selection at a final concentration of 600 μ g/ml was started 36 h later. After ~ 2 weeks with frequent changes of the medium, the selection was complete and we obtained cell lines stably expressing wild-type *RAD51C*, its missense variants or *RAD51B*. These transfectants were exposed to mitomycin C (40 ng/ml) for 15 h. Thereafter, cells were fixed with 100% MeOH for 30 min at -20°C , permeabilized with -20°C , cold acetone for 5 s and washed in PBS. The slides were blocked with 0.5% FCS in PBS for 1 h, exposed to a rabbit polyclonal anti-RAD51 antibody (ab63801; Abcam) at a dilution of 1:800 for 30 min, washed again and exposed to Alexa Fluor® 594 F(ab')₂ fragment of goat anti-rabbit IgG (H + L) (A11072; Invitrogen) at a dilution of 1:2000 in blocking buffer for 30 min. After final washing, the cells were counterstained with 4',6-diamidino-2-phenylindole in Vectorshield Mounting Medium (Vector Laboratories). We determined the percentage of foci-positive cells (more than 10 RAD51 foci per nucleus) visually on a Zeiss Axio Imager A1 fluorescence microscope, thereby assaying the capability of the *RAD51C* variants to restore deficient RAD51 foci formation in the *RAD51C*-mutant cell line. For each experiment, ~ 100 nuclei were analyzed. Recorded was the percentage of RAD51 foci-positive cells for each variant, compared with mock (*RAD51B*)-transfected and wild-type *RAD51C*-transfected cells. Also considered were the number and size of RAD51 foci in positive nuclei.

The functional assay was performed with two other RAD51C-deficient cell lines, the Rad51c-deficient hamster cell line *irs3* (31) treated in the same way or with the human lymphoblast line SH2038-L (c.773G>A, p.R258H) (13) with cells of the latter attached to slides by cyto-centrifugation (data not shown). The results obtained were in agreement with that accrued from studies of SH-2038 fibroblasts, although the latest were the most discriminatory and therefore form the basis of our present study.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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REFERENCES

- Diez, O., Osorio, A., Duran, M., Martinez-Ferrandis, J.I., de la Hoya, M., Salazar, R., Vega, A., Campos, B., Rodriguez-Lopez, R., Velasco, E. *et al.* (2003) Analysis of BRCA1 and BRCA2 genes in Spanish breast/ovarian cancer patients: a high proportion of mutations unique to Spain and evidence of founder effects. *Hum. Mutat.*, **22**, 301–312.
- Osorio, A., Barroso, A., Martinez, B., Cebrian, A., San Roman, J.M., Lobo, F., Robledo, M. and Benitez, J. (2000) Molecular analysis of the BRCA1 and BRCA2 genes in 32 breast and/or ovarian cancer Spanish families. *Br. J. Cancer*, **82**, 1266–1270.
- Antoniou, A.C., Pharoah, P.D., McMullan, G., Day, N.E., Ponder, B.A. and Easton, D. (2001) Evidence for further breast cancer susceptibility genes in addition to BRCA1 and BRCA2 in a population-based study. *Genet. Epidemiol.*, **21**, 1–18.
- de la Hoya, M., Perez-Segura, P., Van Orsouw, N., Diaz-Rubio, E. and Caldes, T. (2001) Spanish family study on hereditary breast and/or ovarian cancer: analysis of the BRCA1 gene. *Int. J. Cancer*, **91**, 137–140.
- Borresen, A.L., Andersen, T.I., Garber, J., Barbier-Piroux, N., Thorlacius, S., Eyfjord, J., Ottestad, L., Smith-Sorensen, B., Hovig, E., Malkin, D. *et al.* (1992) Screening for germ line TP53 mutations in breast cancer patients. *Cancer Res.*, **52**, 3234–3236.
- Lynch, E.D., Ostermeyer, E.A., Lee, M.K., Arena, J.F., Ji, H., Dann, J., Swisshelm, K., Suchard, D., MacLeod, P.M., Kvinnsland, S. *et al.* (1997) Inherited mutations in PTEN that are associated with breast cancer, Cowden disease, and juvenile polyposis. *Am. J. Hum. Genet.*, **61**, 1254–1260.
- Meijers-Heijboer, H., van den Ouweland, A., Klijn, J., Wasielewski, M., de Snoo, A., Oldenburg, R., Hollestelle, A., Houben, M., Crepin, E., van Veghel-Plandsoen, M. *et al.* (2002) Low-penetrance susceptibility to breast cancer due to CHEK2(*)1100delC in noncarriers of BRCA1 or BRCA2 mutations. *Nat. Genet.*, **31**, 55–59.
- Renwick, A., Thompson, D., Seal, S., Kelly, P., Chagtai, T., Ahmed, M., North, B., Jayatilake, H., Barfoot, R., Spanova, K. *et al.* (2006) ATM mutations that cause ataxia-telangiectasia are breast cancer susceptibility alleles. *Nat. Genet.*, **38**, 873–875.
- Seal, S., Thompson, D., Renwick, A., Elliott, A., Kelly, P., Barfoot, R., Chagtai, T., Jayatilake, H., Ahmed, M., Spanova, K. *et al.* (2006) Truncating mutations in the Fanconi anemia J gene BRP1 are low-penetrance breast cancer susceptibility alleles. *Nat. Genet.*, **38**, 1239–1241.
- Rahman, N., Seal, S., Thompson, D., Kelly, P., Renwick, A., Elliott, A., Reid, S., Spanova, K., Barfoot, R., Chagtai, T. *et al.* (2007) PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. *Nat. Genet.*, **39**, 165–167.
- Cybulski, C., Wokolorczyk, D., Jakubowska, A., Huzarski, T., Byrski, T., Gronwald, J., Masojc, B., Deebniak, T., Gorski, B., Blecharz, P. *et al.* (2011) Risk of breast cancer in women with a CHEK2 mutation with and without a family history of breast cancer. *J. Clin. Oncol.*, **29**, 3747–3752.
- Meindl, A., Hellebrand, H., Wiek, C., Erven, V., Wappenschmidt, B., Niederacher, D., Freund, M., Lichtner, P., Hartmann, L., Schaal, H. *et al.* (2010) Germline mutations in breast and ovarian cancer pedigrees establish RAD51C as a human cancer susceptibility gene. *Nat. Genet.*, **42**, 410–414.
- Vaz, F., Hanenberg, H., Schuster, B., Barker, K., Wiek, C., Erven, V., Neveling, K., Endt, D., Kesterton, I., Autore, F. *et al.* (2010) Mutation of the RAD51C gene in a Fanconi anemia-like disorder. *Nat. Genet.*, **42**, 406–409.
- Zheng, Y., Zhang, J., Hope, K., Niu, Q., Huo, D. and Olopade, O.I. (2010) Screening RAD51C nucleotide alterations in patients with a family history of breast and ovarian cancer. *Breast Cancer Res. Treat.*, **124**, 857–861.
- Clague, J., Wilhoite, G., Adamson, A., Bailis, A., Weitzel, J.N. and Neuhausen, S.L. (2011) RAD51C germline mutations in breast and ovarian cancer cases from high-risk families. *PLoS One*, **6**, e25632.
- Akbari, M.R., Tonin, P., Foulkes, W.D., Ghadirian, P., Tischkowitz, M. and Narod, S.A. (2010) RAD51C germline mutations in breast and ovarian cancer patients. *Breast Cancer Res.*, **12**, 404.
- Pang, Z., Yao, L., Zhang, J., Ouyang, T., Li, J., Wang, T., Fan, Z., Fan, T., Lin, B. and Xie, Y. (2011) RAD51C germline mutations in Chinese women with familial breast cancer. *Breast Cancer Res. Treat.*, **129**, 1019–1020.
- Pelttari, L.M., Heikkinen, T., Thompson, D., Kallioniemi, A., Schleutker, J., Holli, K., Blomqvist, C., Aittomaki, K., Butzow, R. and Nevanlinna, H. (2011) RAD51C is a susceptibility gene for ovarian cancer. *Hum. Mol. Genet.*, **20**, 3278–3288.
- Romero, A., Perez-Segura, P., Tosar, A., Garcia-Saenz, J.A., Diaz-Rubio, E., Caldes, T. and de la Hoya, M. (2011) A HRM-based screening method detects RAD51C germ-line deleterious mutations in Spanish breast and ovarian cancer families. *Breast Cancer Res. Treat.*, **129**, 939–946.
- Vuorela, M., Pylkas, K., Hartikainen, J.M., Sundfeldt, K., Lindblom, A., von Wachenfeldt Wappling, A., Haanpaa, M., Puistola, U., Rosengren, A., Anttila, M. *et al.* (2011) Further evidence for the contribution of the RAD51C gene in hereditary breast and ovarian cancer susceptibility. *Breast Cancer Res. Treat.*, **130**, 1003–1010.
- Thompson, E.R., Boyle, S.E., Johnson, J., Ryland, G.L., Sawyer, S. and Choong, D.Y., Kconfab, Chenevix-Trench, G., Trainer, A.H., Lindeman, G.J. *et al.* (2011) Analysis of RAD51C germline mutations in high-risk breast and ovarian cancer families and ovarian cancer patients. *Hum. Mutat.*, **33**, 95–99.
- Somyajit, K., Subramanya, S. and Nagaraju, G. (2012) Distinct roles of FANCO/RAD51C in DNA damage signaling and repair: implications for Fanconi anemia and breast cancer susceptibility. *J. Biol. Chem.*, **287**, 3366–3380.
- Kumar, P., Henikoff, S. and Ng, P.C. (2009) Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat. Protoc.*, **4**, 1073–1081.
- Adzhubei, I.A., Schmidt, S., Peshkin, L., Ramensky, V.E., Gerasimova, A., Bork, P., Kondrashov, A.S. and Sunyaev, S.R. (2010) A method and server for predicting damaging missense mutations. *Nat. Methods*, **7**, 248–249.
- Forget, A.L. and Kowalczykowski, S.C. (2010) Single-molecule imaging brings Rad51 nucleoprotein filaments into focus. *Trends Cell Biol.*, **20**, 269–276.
- Leveille, F., Blom, E., Medhurst, A.L., Bier, P., Laghmani el, H., Johnson, M., Rooimans, M.A., Soback, A., Waisfisz, Q., Arwert, F. *et al.* (2004)

- The Fanconi anemia gene product FANCF is a flexible adaptor protein. *J. Biol. Chem.*, **279**, 39421–39430.
27. Beauchamp, N.J., Taybert, J., Champion, M.P., Layet, V., Heinz-Erian, P., Dalton, A., Tanner, M.S., Pronicka, E. and Sharrard, M.J. (2007) High frequency of missense mutations in glycogen storage disease type VI. *J. Inherit. Metab. Dis.*, **30**, 722–734.
28. Honrado, E., Osorio, A., Palacios, J. and Benitez, J. (2006) Pathology and gene expression of hereditary breast tumors associated with BRCA1, BRCA2 and CHEK2 gene mutations. *Oncogene*, **25**, 5837–5845.
29. de la Hoya, M., Gutierrez-Enriquez, S., Velasco, E., Osorio, A., Sanchez de Abajo, A., Vega, A., Salazar, R., Esteban, E., Llort, G., Gonzalez-Sarmiento, R. *et al.* (2006) Genomic rearrangements at the BRCA1 locus in Spanish families with breast/ovarian cancer. *Clin. Chem.*, **52**, 1480–1485.
30. Gutierrez-Enriquez, S., de la Hoya, M., Martinez-Bouzas, C., Sanchez de Abajo, A., Ramon y Cajal, T., Llort, G., Blanco, I., Beristain, E., Diaz-Rubio, E., Alonso, C. *et al.* (2007) Screening for large rearrangements of the BRCA2 gene in Spanish families with breast/ovarian cancer. *Breast Cancer Res. Treat.*, **103**, 103–107.
31. French, C.A., Masson, J.Y., Griffin, C.S., O'Regan, P., West, S.C. and Thacker, J. (2002) Role of mammalian RAD51L2 (RAD51C) in recombination and genetic stability. *J. Biol. Chem.*, **277**, 19322–19330.