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EPIDEMIOLOGY

A HRM-based screening method detects *RAD51C* germ-line deleterious mutations in Spanish breast and ovarian cancer families

Atocha Romero · Pedro Pérez-Segura · Alicia Tosar · José Ángel García-Saenz · Eduardo Díaz-Rubio · Trinidad Caldés · Miguel de la Hoya

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Abstract The *RAD51C* gene has been recently proposed as a high-penetrance breast and ovarian cancer gene. However, early replication studies have failed to confirm the finding. Thus, further studies in larger cohorts should be conducted in order to clarify the role of *RAD51C* as a cancer susceptibility gene. Here, we describe a high-resolution melting analysis (HRMA)-based method developed for presequence screening of *RAD51C* sequence variants. We have screened *RAD51C* sequence variants by HRMA in 492 breast cancer patients with family history of breast and/or ovarian cancer that were previously tested negative for *BRCA1/2*. All variants were confirmed by direct sequencing. We have detected 12 different *RAD51C* germ-line sequence variants, including eight transitions, two transversion, and two indels (insA, and delT). All these variants generated melting profiles which differ from wild type homozygous controls. Interestingly, we have identified one clearly pathogenic mutation (c.774delT) in the subset of 101 breast and ovarian cancer families, supporting that *RAD51C* is a human breast and ovarian cancer susceptibility gene.

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A. Romero · A. Tosar · E. Díaz-Rubio · T. Caldés (✉) · M. de la Hoya (✉)
Laboratorio de Oncología Molecular, Planta baja sur. Hospital Clínico San Carlos, c/Martín Lagos s/n, 28040 Madrid, Spain
e-mail: tcaldes.hcsc@salud.madrid.org

M. de la Hoya
e-mail: mdhoya@hotmail.com

P. Pérez-Segura · J. Á. García-Saenz · E. Díaz-Rubio
Servicio de Oncología Médica, Hospital Clínico San Carlos, Madrid, Spain

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Introduction

Family history is a well-established risk factor for breast cancer development. Germ-line deleterious mutations in the breast cancer susceptibility genes *BRCA1* (OMIM 113705) and *BRCA2* (OMIM 600185) explain approximately one-half of all hereditary breast cancer cases attending familial cancer clinics. The risk of breast cancer in *TP53* (OMIM 191770) and *PTEN* (OMIM 601728) mutation carriers is very high. However, germ-line alterations in these genes do not cause hereditary breast cancer syndrome, but *Li-Fraumeni* syndrome and Cowden disease [1, 2]. Other breast cancer susceptibility genes such as *CHEK2* (OMIM 604373), *BRIP1* (OMIM 605882), and *PALB2* (OMIM 610355) are considered moderate penetrance genes not causing hereditary syndromes [3–5]. Nevertheless, the proportion of breast cancer families that can be explained by these genes is very low. Numerous studies have been conducted in order to identify breast cancer hereditary syndrome genes others than *BRCA1/2*, although with little success [6, 7].

Recently, Meindl et al. identified biallelic *RAD51C* mutations in a Fanconi anemia-like disorder [8]. Since monoallelic mutations in some Fanconi anemia (FA) genes increase breast cancer risk, [4, 5] the authors investigated whether monalleic mutations in *RAD51C* confer such phenotype. For that purpose, they screened *RAD51C* germ-line nucleotide alterations in 1,100 unrelated affected women from pedigrees with hereditary breast and/ovarian cancer that were previously negative for mutations in *BRCA1/2*, identifying deleterious mutations segregating in

six families [9]. Their data suggest that *RAD51C* might be a truly high-penetrance breast and ovarian cancer gene resembling *BRCA1/2* in that nearly complete segregation is observed. Interestingly, they identified all deleterious mutations in the subset of 480 families with both breast and ovarian cancer cases [9]. Two replication studies were soon published [10, 11]. Both failed to identify clearly deleterious *RAD51C* germ-line alterations. Thus, further studies in larger cohorts of different ethnicities should be conducted to best characterize the role of *RAD51C* as a breast/ovarian cancer susceptibility gene. Moreover, in order to estimate the risk conferred by this gene, and thus the clinical implications of carrying a germ-line mutation, it is mandatory to identify a large number of *RAD51C* families. To facilitate these studies, we have developed a *RAD51C* presequence screening method based on high resolution melting analysis (HRMA).

In the present study, we describe the method, and we report the identification of a novel *RAD51C* deleterious mutation (c.774delT, p.Arg258fs) in one out of 492 Spanish breast and/or ovarian cancer families investigated, thus replicating previous findings by Meindl et al. [9].

Materials and methods

Study population and DNA samples

492 unrelated women attending the familial cancer clinic at Hospital Clinico San Carlos, Madrid, Spain from 1998 to 2010, who were referred for *BRCA1* and *BRCA2* genetic testing due to a personal and family history of breast and/or ovarian cancer compatible with a hereditary syndrome. Most women reported Spanish ancestry, although various other ancestries are also present in our cohort. The cohort included:

- Women diagnosed of breast cancer at age 35 or younger with no family history ($N = 34$). Most of these women were selected for genetic testing because of their limited family structures [12].
- Women diagnosed of breast and/or ovarian cancer at age 50 or younger, with one first degree relative diagnosed of breast and/or ovarian cancer at any age ($N = 168$). In 34 of these families, at least one ovarian cancer case was present.
- Women diagnosed of breast and/or ovarian cancer at age 50 or younger, with a minimum of two additional breast and/or ovarian cancer cases diagnosed at any age in two generations of the same parental branch ($N = 290$). In 67 of these families, at least one ovarian cancer was present.

After comprehensive *BRCA1* and *BRCA2* genetic testing (full coding sequence, intron/exon boundaries, and genomic rearrangements), all 492 women were considered negative for the presence of germ-line mutations [13–15].

DNA was obtained from blood leukocytes with MagNA Pure LC Total Nucleic Acid Extraction kit and a MagNA Pure LC instrument (Roche Diagnostics, Penzberg, Germany). All samples were diluted with deionised water to a final concentration of 25 ng/µl.

The study was approved by the Ethics Committee of Hospital Clinico San Carlos, Madrid and all patients signed an informed consent to participate in the study.

HRMA-based *RAD51C* presequence screening

RAD51C specific primers were designed with Primer3 software with default settings [16]. We designed amplicons in the 150–250 bp size range, as amplicons of this size are expected to perform well in HRMA-based presequence screening [17]. Our method targets the 9 *RAD51C* coding exons present in Ensemble transcript ID INST00000337432 (1,131 bp of coding sequence) plus the corresponding exon/intron boundaries. We used GRCh37 (Ensemble release 60) as the reference sequence for the *RAD51C* genomic region (GRCh37:17:56769334:56812303:1). To avoid primers overlapping polymorphic sites, we imported from dbSNP 131 all SNPs and indels reported in CEU population (May 2010).

Initially, 12 *RAD51C* specific amplicons were designed. For exons 1, 4, 6, 7, 8, and 9 we used single amplicons, while exons 2, 3, and 5 were divided into two overlapping fragments in order to obtain PCR products of the desired size. *In silico* primers specificity was checked with Basic Local Alignment Search Tool (BLAST) form NCBI [18].

PCR amplification and high resolution melting analysis (HRMA) were carried out in a LightCycler 480 Instrument (Roche Diagnostics, Penzberg, Germany) with a 96-well thermal block. All assays were performed in 20 µl final volume reaction. Optimal PCR annealing temperatures (T_a) and $MgCl_2$ concentrations were evaluated for each amplicon. In optimal conditions, agarose gel electrophoresis revealed the presence of a single PCR product of the expected size and no primer-dimmers. Direct sequencing confirmed specificity. In brief, optimal PCR reactions contained 25 ng of genomic DNA, forward and reverse HPLC purified primers 0.5 µM each, 3 mM $MgCl_2$ (6 mM for amplicon 9), and 1× LightCycler 480 High Resolution Melting Master Mix (Roche Diagnostics, Penzberg, Germany). Thermal cycling consisted of an initial 10-min hold at 95°C, followed by 10-second hold at 95°C, 10-second hold at the indicated T_a (Table 1), and 10-second hold at 72°C for 45 cycles. Finally, heteroduplexes were generated by adding a step at 95°C for 1 min and cooling the reaction to 40°C (Ramp rate of 2.2°C/s).

Table 1 *RAD51C* HRM primers

Exon	Amplicon	Forward primer	Reverse primer	Size (bp)	T_a (cycles)[a]
1	1	CCGGGGTTAGCAGGTGAG	AGGCGAGAGAACGAAGA	247	60 (45)
2	2A	TGTTTCTCCACTCCTAGCATCA	GCTCCTGCTCAAGAAGTTCC	253	62 (25) + 58 (20)
	2B	TGCTGGTACATCTGAGTCACAC	CCCACCCTTAAAAGGAGAACA	212	60 (45)
3	3A	TCATGATTTGGTTGTTTGTCATC	AATGCAGGCAGTAGCAAGGT	163	65 (45)
	3B	GCAGTTGGCAGTAGATGTGC	AGGTCTCAGATGGGCACAAA	236	65 (45)
4	4	TGCCAATACATCCAAACAGG	CTTGGTTAGTTACATTTCAGTAGTCTG	221	60 (45)
5	5A	AACAAATCTAATATTATCTCTTCTGTA	TGATTATTTGCAAGGCTGAT	155	60 (45)
	5B	GGATGGTATTGCTTTTCCATT	TCAGGCAAACGCTATTTTGA	206	62 (25) + 58 (20)
6	6	ATGGGGTTTCACAATCTTGG	TCAATGAGAATCAAATGAAAGAGA	225	60 (15) + 55 (30)
7	7	CCAAGTCAGTAAGGCCATATACA	CCACAGGACTAGCTCTAAGAAACC	195	62 (45)
8	8	AATGAGTTTGGTCATCTGAACTTTT	CCGCCCATCAATATCAAAAT	182	60 (45)
9	9	TCAGTCTTCAAATGTTCTTAAAGC	CCACTTGTACACATTGATTTCACA	222	60 (45)

[a] Optimal annealing temperature (number of PCR cycles)

HRMA was performed in a 65 to 95°C melting gradient with a ramp rate of 0.04°C/s and continuous acquisition mode set at 25 acquisitions/°C, regardless of the amplicon analyzed. Melting curves were analyzed with LightCycler 480 Software version 1.5 which automatically normalized the melting curves. Temperatures at where initial and final fluorescence is set, which are used to normalize data, were checked by visualizing Melting Curves. Manual inspection of Normalized Melting Curves suggested that *RAD51C* amplicons performed well in HRM (Supplemental Data Figure 1). The only exception was amplicon 5A, for which a high dispersion was observed among samples (Supplemental Data Figure 2A). In our experience with HRMA, such dispersion is characteristic of microsatellite-containing fragments. Although Amplicon 5A does not contain any obvious microsatellite, we observed 3 short poly(dT) tracts clustered together at the 3′ end of intron 4 (Supplemental Data Figure 2). To avoid these repetitive sequences, we designed a new forward primer closer to exon 5. The new Amplicon 5A performed well and dispersion was suppressed (Supplemental Data Figure 2B). As shown in Supplemental Data Figure 1, amplicon 9 contains 3 melting domains, a circumstance which may decrease sensitivity [17]. All primer sequences are detailed in Table 1.

Samples were screened by direct comparison of the Normalized and Temp-Shifted Melting Curves. All samples with curves that differed in shape and/or melting temperature were considered as potential variant carriers and were directly sequenced for confirmation.

Sequencing

Samples were sequenced using the Big Dye Terminator v1.1 Cycle Sequencing Reaction kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. All sequence variants are named according to Human Genoma Variation Society (HGVS) nomenclature, using Ensemble transcript ID ENST00000337432 as a reference sequence.

In silico predictions

The severity of *RAD51* missense changes was analyzed with PolyPhen [19]. Splice site analysis was performed with Human Splicing Finder [20]. In both cases, default settings were used.

Results

We have performed HRM-based *RAD51C* mutation scanning in 492 breast and/or ovarian patients attending the familial cancer clinic of our Hospital. In total, we have detected 12 different *RAD51C* germ-line sequence variants (Table 2), including 8 transitions (2 G>A, 3 A>G, and 3 T>C), two transversion (1 C>A, and 1 G>T),and two indels (insA, and delT). All sequence variants generated melting profiles which differ from wt homozygous controls both in melting temperature (T_m) and shape (Fig. 1, Supplemental Data Figures 1, 2, and 3). Moreover, if two or more variants were located in the same amplicon, different heterozygous genotypes produced clearly distinguishable melting profiles (Supplemental Data Figures 1 and 3). Amplicon 6 is of particular interest to show sensitivity and specificity of the assay. This amplicon permitted us to genotype the c.904+34T>C variant (rs28363318), since homozygous genotypes are easily discriminated (Supplemental Data Figure 3A). This variant is a common SNP in CEU population (MAF = 0.33). Genotype frequencies

Table 2 Germ-line *RAD51C* sequence variants identified in 492 Spanish breast and/or ovarian cancer families

Location	Sequence variant		Effect	Previously described?[a]	*N*	Family phenotype[b]	MAF[c]	
	Nucleotide	Protein					This study	CEU
Ex 1	c.89C>A	p.Ala30Glu	Unknown	Novel	1	BC		–
Ex 1	c.106G>A	p.Glu36Lys	Unknown	Novel	1	BC		–
Ex 1	c.134A>G	p.Glu45Gly	Unknown	Novel	1	BC		–
IVS 1	c.146-67 dupA	Intronic	Unknown	Novel	1	BOC		–
Ex 2	c.376G>A	p.Ala126Thr	Unknown	rs61758784	2[d]	BC	0.002	n.d.[e]
Ex 3	c.428A>G	p.Gln143Arg	Unknown	Novel	1	BC		–
IVS 3	c.572-17G>T	Intronic	Unknown	novel	4	BC	0.004	–
Ex 4	c.586T>C	p.Leu196Leu	Unknown	Novel	1	BC		–
Ex 5	c.774delT	p.Arg258fs	Deleterious	Novel	1	BOC		–
Ex 6	c.859A>G	p.Thr287Ala	Unknown	rs28363317	15	BC/BOC	0.015	0.017
Ex 6	c.869T>C	p.Ile290Thr	Unknown	Novel	1	BC		–
IVS 6	c.904+34T>C	Intronic	Neutral	rs28363318	312	BC/BOC	0.390	0.33

[a] dbSNP 130 (accessed 16/12/2010) and/or bibliography [9–11]

[b] Breast cancer only (BC), at least one ovarian cancer (BOC)

[c] Minor allele frequency not displayed for singletons

[d] Carry c.572-17G>T

[e] No frequency data

were consistent with Hardy–Weinberg equilibrium (data not shown), supporting calling accuracy. Moreover, a second SNP (rs28363317) is detected with this amplicon. Heterozygous genotypes and double heterozygous genotypes are easily discriminated from each other (Supplemental Data Figure 3B). Finally, a new *RAD51C* variant was identified (c.869T>C), despite the presence of the two polymorphisms (Supplemental Data Figure 3C).

Among the 12 *RAD51C* sequence variants identified in this study, nine represent novel changes, eight of them observed only once (i.e., singletons) (Table 2). With the exception of c.774delT (p.Arg258fs), a clearly deleterious mutation leading to frame-shift and premature protein termination, these novel variants should be considered of uncertain clinical significance. The c.774delT deleterious mutation was identified in a woman diagnosed of breast cancer at age 38. A moderated history of breast and ovarian cancer was observed both in the paternal and maternal branches (Fig. 1). Her father was Peruvian and her mother Swedish. We could genotype both parents. The mother, healthy at age 70, carried the mutation.

We have identified in dbSNP130 (accessed 16/12/2010) 15 *RAD51C* sequence variants located in the region targeted by our assay. The scientific literature [9–11] reports 13 additional variants located in this region, most of them singletons. Only 3 of these 28 previously reported variants (rs61758784, rs28363317, and rs28363318) have MAF >0.005 in European ancestry populations (Table 3). We have detected all 3 variants in our cohort, supporting the sensitivity of the HRMA assay.

Discussion

The study by Meindl et al. [9] supporting a role for *RAD51C* in human breast and ovarian cancer susceptibility was conducted in 1,100 breast/ovarian families from Germany. Soon, replication studies conducted in 92 families recruited in Chicago, [10] and 480 families from Canada (100 patients reported to be Jewish, 152 French–Canadian, and 202 of various other ethnic origins) [11] were reported. None of these two replication studies was able to confirm *RAD51C* as a breast and ovarian cancer susceptibility gene, as they did not detect any obvious deleterious mutation. In all three studies, *RAD51C* mutation screening was performed by direct sequencing.

Based on its low cost, high-throughput, nondestructive nature, sensitivity, and specificity, HRMA is an increasingly popular technology to screen individuals for sequence variants [21–24]. In the present manuscript, we report the development of a HRMA-based presequence screening method which might facilitate large *RAD51C* mutation scanning efforts. Such studies are mandatory to assess the mutational frequency of this gene in different populations, as well as the clinical implications of carrying a *RAD51C* germ-line mutation.

Overall, the *RAD51C* HRMA method we have developed seems to perform well, although the possibility exists that certain *RAD51C* variants are not detected. In particular, we should be cautious with exon 9 analysis since, in fragments contain more than two melting domains chances increase that not all variants are detected.

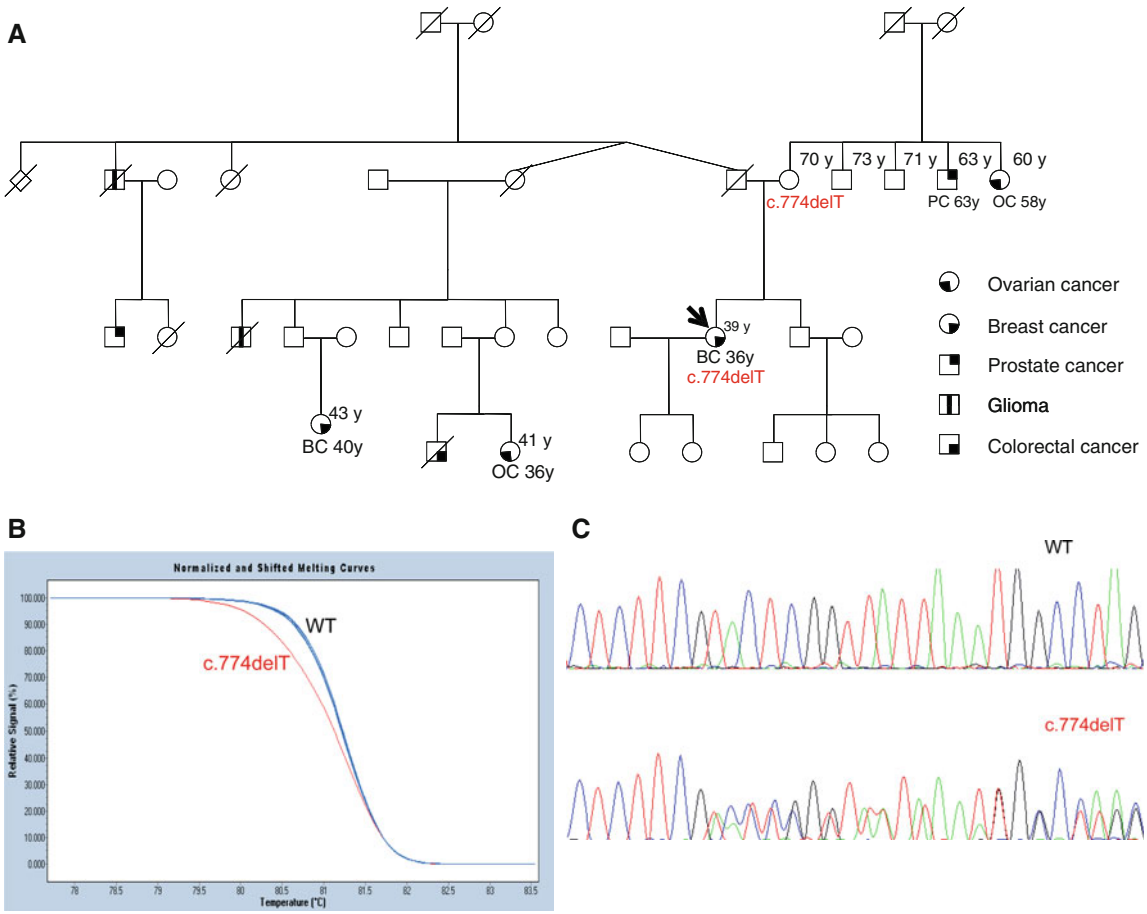

Fig. 1 Identification of a *RAD51C*-related breast/ovarian cancer family. **a** The *RAD51C*-related pedigree identified in this study. Both the index case (indicated by an arrow) and her mother carry the germ-line mutation. Nobody else has been tested. **b** *RAD51C* c.774delT detection by HRMA. **c** Sequence characterization of the germ-line mutation

We have tested our method by conducting a search for *RAD51C* germ-line mutations in 492 breast and/or ovarian cancer patients with a family history of breast/ovarian cancer but without any *BRCA1/2* mutation detected after comprehensive screening. Of these, only 101 patients have a family history of both breast and ovarian cancer, including 34 patients with only one affected female relative. The data is relevant since *RAD51C* was initially described by Meindl et al. as a predisposing gene for breast and ovarian cancer families but not for breast only families.

Overall, we have identified one clearly deleterious mutation (c.774delT) in 492 families (0.2%). Meindl et al. identified 3 such mutations (c.224_225insA, 525_526insC, and c.145+1G>T) in 1,100 families (0.3%). As noted, Meindl et al. found all 3 mutations in the subset of 480 families with at least one ovarian cancer (0.6%), suggesting that *RAD51C* is an ovarian cancer susceptibility gene. Similarly, we have found the c.774delT mutation in the subset of 101 breast and ovary cancer families (1%). However, the mutation has not been tested in the ovarian cancer case, so the possibility of a phenocopy does exist.

A combination of functional analysis permitted Meindl et al. [9] to identify 3 additional pathogenic mutations (c.904+5G>T, p.Gly125Val, and Leu138Phe), all of them in families with at least one ovarian cancer reported. We have detected ten variants that should be considered of uncertain significance (Table 2). Two variant, p.Ala126Thr and p.Thr287Ala, might be benign, as suggested by lack of association with cancer in case/control studies [9]. The remaining eight variants represent novel findings. At present, we cannot rule out that some of these variants represent true *RAD51C* deleterious mutations. In particular, p.Ala30Glu and p.Gln143Arg may have some clinical relevance, as they target evolutionally conserved residues (supplemental table 1). The p.Ala30Glu was identified in a breast cancer female, but diverse cancer cases were observed both in the maternal and parental branches. Interestingly, the p.Gln143Arg (a putative pathogenic mutation) was identified in a breast cancer only family (supplemental figure 4). Further studies are required to determine in what extent *RAD51C* mutations might occur in this type of families.

Table 3 Identification of *RAD51C* variants previously reported in other studies

Amplicon	Sequence variant	MAF[a]							
		CEU[b]	YRI[b]	CHB[b]	PDR90[b]	[ref 9]	[ref 10]	[ref 11]	This study
1	c.7G>A	–	–	–	–	(1)[c]	–	–	–
	c.34C>T (rs28910276)	0.005	<0.005	<0.005	<0.005	–	–	–	–
	c.145+1G>T	–	–	–	–	(1)[c]	–	–	–
2A	c.146-8A>G	–	–	–	–	–	–	0.031(0.015)[d]	–
	c.150T>G (rs1127858)	n.r	n.r	n.r	n.r	–	–	–	–
	c.186A>G (rs28363303)	n.r	n.r	n.r	0.006	–	(1)[c]	–	–
	c.195A>G (rs45511291)	n.r	n.r	–	–	–	(1)[c]	–	–
	c.224_225insA	–	–	–	–	(1)[c]	–	–	–
2B	c.374G>T	–	–	–	–	(1)[c]	–	–	–
	c.376G>A (rs61758784)	n.r	n.r	n.r	n.r	0.006(0.007)	–	(0.003)	(0.002)
3A/3B	c.414G>C	–	–	–	–	(1)[c]	–	–	–
	c.431T>C (rs28363307)	n.r	n.r.	n.r	0.006	–	–	–	–
	c.453G>A (rs45553636)	n.r	n.r.	n.r.	n.r.	–	–	–	–
	c.459T>G (rs74885602)	0.07[e]	n.r	n.r	n.r	–	–	–	–
	c.475G>A	–	–	–	–	(1)[c]	–	–	–
	c.485G>A (rs35151472)	n.r	n.r	n.r	n.r	–	–	–	–
	c.506T>C	–	–	–	–	(1)[c]	–	–	–
3B	c.525_526insC	–	–	–	–	(1)[c]	–	–	–
4	Not reported								
5A/5B	c.745C>T (rs28363311)	n.r	n.r	n.r	0.007	–	–	–	–
	c.790G>A	–	–	–	–	0.003(0.005)	–	–	–
	c.791G>T	–	–	–	–	(1)[c]	–	–	–
5B	c.837+39C>G (rs34554540)	n.r.	n.r.	n.r.	n.r.	–	–	–	–
6	c.859A>G (rs28363317)	0.015	<0.005	<0.005	0.006	0.007(0.006)	(0.022)	(0.004)	(0.015)
	c.900A>T (rs78702006)	n.r.	n.r.	0.011	n.r.	–	–	–	–
	c.904+5G>T	–	–	–	–	(1)[c]	–	–	–
	c.904+34T>C (rs28363318)	0.33	0.20	n.r.	0.19	–	(0.37)	–	(0.39)
7	Not reported								
8	Not reported								
9	c.1097G>A	–	–	–	–	(1)[c]	–	–	–
	c.*25C>G (rs28363336)	–	–	–	0.028	–	–	–	–

n.r. not reported

[a] Minor allele frequency in healthy controls or (Breast/Ovarian cases)

[b] db SNP131 (December 2010)

[c] Singleton variants

[d] Jewish specific

[e] $N = 30$ individuals from pilot 1 CEU-The 1000 genomes project

Interestingly, the only *RAD51C* clear deleterious mutation that we have found was of Swedish origin (a genetic background very uncommon in the Spanish population and not represented by any other individual in our cohort), so that the detection of a deleterious mutation may represent serendipity finding in an otherwise *RAD51C* negative Spanish cohort. It will be interesting to investigate the prevalence of *RAD51C* c.774delT in Swedish breast/ovarian families.

The c.774delT mutation was identified in a pedigree with breast, ovarian, and prostate cancer (at present, the mutation status is unknown both in the women diagnosed of ovarian cancer and the male with prostate cancer). In the six *RAD51C* families reported by Meindl, apart from breast and ovarian cancer, other malignancies such as lung, kidney, pancreatic, and colon cancer were apparent. However, prostate cancer was not present in any of these six families. Only the identification of a much larger set of *RAD51C*

families will permit to characterize the cancer spectrum associated with these mutations.

In conclusion, we have developed a HRMA-based *RAD51C* presequence screening method. The method has permitted us to genotype previously described SNPs and to identify nine novel *RAD51*C germ-line variants, including a clearly deleterious mutation identified in a breast and ovarian cancer family, thus replicating for the first time previous findings by Meindl et al. We hope that this method will help laboratories worldwide to explore the role of *RAD51C* germ-line mutations in cancer susceptibility.

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Conflict of interests None.

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