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Original Article

Germline *RAD51C* mutations in ovarian cancer susceptibility

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Several genes might explain *BRCA1/2* negative breast and ovarian family cases. Deleterious mutations in few genes involved in the Fanconi complex are responsible for Fanconi anemia at the homozygous state and breast cancer (BC) susceptibility at the heterozygous state (*BRCA2, PALB2, BRIP1*). *RAD51C* plays an important role in the double-strand break repair pathway and a biallelic missense mutation in the *RAD51C* gene was found in a Fanconi anemia-like disorder. Subsequently, six monoallelic pathogenic mutations were identified after screening 480 *BRCA1/2* negative breast and ovarian cancer (BC/OC) pedigrees. Several reports were unsuccessful to replicate these results. To investigate whether germline mutations in *RAD51C* are associated with an increased risk of developing BC/OC, we screened, by Sanger sequencing of the coding sequence, 117 index cases of breast and ovarian families from French or European origin, and negative for *BRCA1/2* mutations. In our study, we found 3 pathogenic mutations among 117 families screened which corresponds to a 2.6% frequency. Our results confirm that *RAD51C* is a susceptibility gene for ovarian and BC and that this gene should be screened for mutations in families with multiple BC/OC.

Conflict of interest

Nothing to declare.

F Coulet[a], A Fajac[b], C Colas[a], M Eyries[a], A Dion-Minière[a], R Rouzier[c], S Uzan[c], J-P Lefranc[d], M Carbonnel[e], F Cornelis[f], A Cortez[g] and F Soubrier[a]

[a]Groupe hospitalier Pitié-Salpêtrière, Assistance Publique-Hopitaux de Paris, Université Pierre et Marie Curie, Département de Génétique, Paris F-75651, France, [b]Hôpital Tenon, Assistance Publique-Hopitaux de Paris, Laboratoire d'Histologie Biologie Tumorale, Paris F-75651, France, [c]Hôpital Tenon, Assistance Publique-Hopitaux de Paris, Service de Chirurgie Gynecologique, Paris F-75651, France, [d]Groupe hospitalier Pitié-Salpêtrière, Assistance Publique-Hopitaux de Paris, Université Pierre et Marie Curie, Service de Chirurgie Gynecologique, Paris F-75651, France, [e]Hôpital Jean Verdier, Assistance Publique-Hopitaux de Paris, Service de Chirurgie Gynecologique, Bondy F-93143, France, [f]Hôpital Jean Verdier, Assistance Publique-Hopitaux de Paris, Service d'Anatomo-pathologie, Bondy F-93143, France, and [g]Hôpital Tenon, Assistance Publique-Hopitaux de Paris, Service d'Anatomo-pathologie, Paris F-75651, France

This paper is dedicated to the memory of Nathalie Davids who actively participated in this work.

Key words: breast cancer susceptibility – *RAD51C* – ovarian cancer susceptibility – germline mutation

Corresponding author: Dr Florence Coulet, Laboratoire d'Oncogénétique et Angiogénétique moléculaire, Bât. 10 La Peyronie, Groupe Hospitalier Pitié-Salpêtrière, 47-83 Bd de l'Hôpital, 75651 Paris cedex 13, France.
Tel.: (33-1) 42 17 76 64;
fax: (33-1) 42 17 76 18;
e-mail: florence.coulet@psl.aphp.fr

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Inherited breast and ovary cancers (BC/OC) as well as other forms of complex disease in which genetic factors play a major role, can be explained by highly penetrant mutations probably located on a large variety of genes, each accounting for a small proportion of cases (1). Therefore, beside major genes such as *BRCA1* and *2*, which account for more than 80% of families with BC/OC, and probably around 25% of families with breast cancer (BC) alone, several other genes might explain the remaining family cases. The genes belonging to the Fanconi complex, to which *BRCA2* belongs, and which are responsible for Fanconi disease at the homozygous state, are candidate genes for predisposition to BC/OC. Indeed, *PALB2*, *BRIP1*, *RAD51C*, *RAD51D* were shown to be mutated in BC/OC predisposition (2–4) but account for a very limited proportion of cases (less than 1%) (5).

In a first article, Meindl et al. (6) have screened a large series of BC ($n = 1100$) and OC ($n = 480$) families for mutation in the *RAD51C* gene, which belongs to the Fanconi Anemia protein complex. They reported six pathogenic germline mutations of *RAD51C*, two insertions, two splice site mutations and two missense alterations. Interestingly, all six mutations were found in OC families. Several reports were unsuccessful to replicate these results. Indeed, a total of 616 families with BC/OC were reported negative for *RAD51C* mutations in different publications (5, 7, 8). A series of 188 families with BC and/or OC, but for which accurate clinical data are not available, were also found negative for *RAD51C* (9). A single *RAD51C* pathogenic mutation was reported after screening 492 families with BC/OC (10). More recently, Pelttari et al. (11) found two recurrent deleterious mutations of *RAD51C* in the Finnish population, which result from a founder effect. These recurrent mutations were associated with a high relative risk for familial OC, but their frequency was not statistically different in BC patients and controls. A recent article reported a frequency of 0.6% of *RAD51C* truncating mutations in families with OC (12).

We report the screening of the *RAD51C* gene in 117 families including at least one case of OC associated or not with BC. We found three pathogenic mutations, a result which strengthens the role of *RAD51C* as a susceptibility gene for OC. Our results show that *RAD51C* is a susceptibility gene for OC, justifying its investigation in BRCA1/2 negative families with OC.

Materials and methods

Patients

Families with at least one case of OC were included in the study (Table 1). Patient peripheral blood samples were collected in ethylene diamine tetraacetique (EDTA) tubes from patients who had previously signed an informed consent: 117 affected index cases were selected. All patients were found negative for BRCA mutation by high resolution melting (HRM) screening, Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA) for the search of large rearrangements (13).

Table 1. Characteristics of the 117 index cases screened

Age at first diagnosis (OC or BC) (years mean ± SD)	49.6 ± 12.3
Gender (female/male)	117/0
Isolated OC	27
OC and relatives with OC	8
OC and relatives with BC	34
BC and relatives with OC	48
Family history of male BC	3
Number of families with other malignancies	61

OC, ovarian cancer; BC, breast cancer.

Nomenclature

The DNA mutation numbering is based on the cDNA sequence for *RAD51C* (GenBank: NM_058216.1). The nomenclature system for the description of changes in DNA, RNA and protein follows the recommendations of the Human Genome Variation Society.

RAD51C amplification and sequencing

DNA was isolated using an automatized Extragene® extractor (Genomic Industry, Archamps, France) with a Wizard® Genomic DNA extraction kit (Promega France, Charbonnières-les-Bains, France) according to the manufacturer's standard protocol. Amplification of the entire sequence and intronic junctions of the *RAD51C* gene was performed on genomic DNA from 117 index cases. Primers and PCR conditions are available on request. Sequencing was performed with Big Dye Terminators v1.1 kit (Applied Biosystems, Courtaboeuf, France) using a DNA sequencer ABI 3730.

Splicing predictions

Potential splice variants identified were analyzed by five splice-site predictions programs: Splice Site Finder, MaxEntScan, Nnsplice and Human Splicing Finder, available through ALAMUT v2.0 software (Biointeractive Software, Rouen, France).

In vitro splicing assay using a splicing reporter minigene

Splice variants were studied in minigene constructions with pSPL3 plasmid. The *RAD51C* exon 7 was PCR-amplified together with about 150 bp of 5′ and 3′ flanking intronic sequences, by using forward and reverse primers carrying 5′ tails that contained restriction sites for *Bam*HI and *Eco*RI, respectively (primer sequences are available upon request). Using these restriction sites, we cloned the fragment in the splicing reporter minigene pSPL3. Minigenes carrying the wild-type (wt) and the mutant allele were then identified by sequencing and were transfected separately into HeLa cells during the

Table 2. Summary of germline RAD51C variants

Location	Nucleotide change	Effect on RNA	Effect on protein	dbSNP	Variation type
5′ UTR	c.1-26C>T	–	–	rs12946397	Polymorphism
ex2	c.376G>A	–	p.Ala126Thr	rs61758784	Polymorphism
ex4	c.577C>T	–	p.Arg193X	Not reported	Nonsense
ex5	c.790G>A	–	p.Gly264Ser	Not reported	Missense
ex6	c.859A>G	–	p.Thr287Ala	rs28363317	Polymorphism
ex6	c.904+34T>C	–	–	rs28363318	Polymorphism
ex7	c.905-2A>G	exon7 skipping	p.Glu303TrpfsX41	Not reported	Splicing mutation
ex7	c.965+5G>A	exon7 skipping	p.Glu303TrpfsX41	Not reported	Splicing mutation

same experiment. The splicing patterns corresponding to the wt and to the mutant allele were then compared by reverse transcription polymerase chain reaction (RT-PCR) analysis of the RNA from transfected cells, using primers positioned in the exons of the pSPL3 minigene and by sequencing all RT-PCR products. Total RNA was isolated from transfected cells or from whole blood samples with the PAXGene Blood RNA system (Qiagen, Courtaboeuf, France), 500 ng of RNA were used for RT-PCR according to manufacturer's recommendations (Superscript II, random primers, Invitrogen, Gif sur Yvette, France).

Results

We studied both families ($n = 90$) and isolated index cases with OC ($n = 27$). The probands studied in the families were either the OC-affected patients ($n = 69$) or a relative with BC ($n = 48$) (Table 1).

All variants identified in this study were reported in Table 2. Four polymorphisms c.1-26C>T, c.376G>A p.Ala126Thr, c.859A>G p.Thr287Ala and c.904+34T>C were identified regularly and previously reported in the literature (5–7, 10). We detected the rare missense variant c.790G>A (p.Gly264Ser). The same variant had also been previously detected by Meindl et al. (6), by Pelttari (11) and by Thompson (12) in cases and population controls.

Mutation screening revealed three mutations: one nonsense mutation c.577C>T (p.Arg193X), two novel splice mutations flanking exon 7, one donor mutation at position +5 (c.965+5A>G), and one acceptor at position −2 (c.905-2A>C) in three families affected by OC (pedigrees shown on Fig. 1a–c). *In silico* predictions were obtained for the two potential splice mutations: for c.965+5G>A the donor site score was diminished (57% MaxEntScan) and for c.905-2A>G, the acceptor site was abolished (MaxEntScan). Either the wt exon 7 and its flanking regions, or the two mutated sequences were introduced into the pSPL3 splicing reporter plasmid, and transfected into HeLa cells. The RT-PCR analysis of the transfected cells showed that both mutations induced loss of exon 7 in the mature transcript, and introduced a frameshift and a stop codon at position 363 (Fig. 2a). These results were confirmed by RT-PCR of the mRNA isolated from blood cells of one index case (no RNA was available for the second case) (Fig. 2b). The three patients carrying *RAD51C* deleterious mutations had developed an OC at 73 (A), 42 (B) and 51 (C)years old: in two cases (A and B), the histological types were invasive serous OC, and was an invasive endometrioid adenocarcinoma in the third case (C). Families 1A and 1C are breast and ovarian families but we were not able to have access to other subjects of the family to study cosegregation (because of death or no response to our invitation of the relatives). In family 1C, the maternal uncle had a BC. No BC was observed in the family but one case of lower abdominal tumor was reported.

Discussion

By identifying inactivating mutations in three index cases from families with OC among 117 screened for *RAD51C* mutation, we confirm data from Meindl et al. (6). showing that *RAD51C* deleterious mutations are found in BC/OC families. In our series, all three mutations were found in OC cases, and no mutation was found in BC index cases even if OC were observed in these families.

The rate of mutation is the most elevated among all published series (2.5%), but close to the rate found in Finnish families with OC cases, where a founder effect was described for the two *RAD51C* mutations identified (11).

The histological type of OC in the patients with *RAD51C* mutation is similar to what is found in BRCA1/2 carriers, with two invasive serous ovarian adenocarcinomas and one endometrioid adenocarcinoma. Of note, a BC in a male patient was observed in a mutation-positive family, but the presence of the mutation could not be verified in this patient. No similar observation was reported, in particular in the study of Silvestri et al. (14) who analyzed 98 male BC patients without BRCA1/2 mutation.

The penetrance of the *RAD51C* mutations cannot be assessed accurately through our study because of the ascertainment bias and because it was not possible to test relatives for the presence of the mutation. In the three families positive for *RAD51C* mutation, it seems likely that there are unaffected carriers although the mutation could not be tested in the putative obligatory carriers. This would confirm the results of Pelttari et al. (11) who found that *RAD51C* mutations were mostly

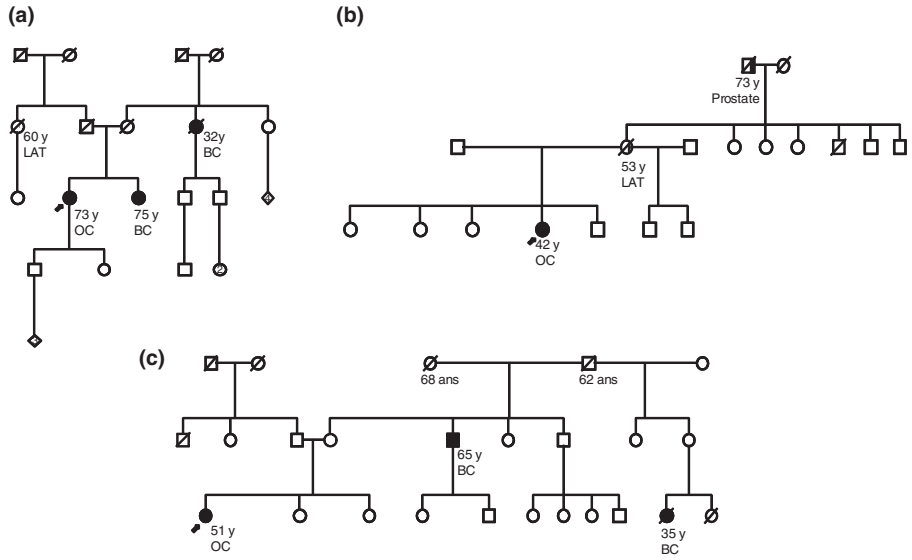

Fig. 1. *RAD51C* mutations in familial breast and ovarian cancer (BC/OC) pedigrees. Individuals with BC or OC are shown as filled circles, and individuals with lower abdominal tumor (LAT) are shown as demi-filled circles. (**a**) Family with c.577C>T (p.Arg193X) mutation (**b**) Family with c.905-2A>G mutation (**c**) Family with c.965+5G>A mutation.

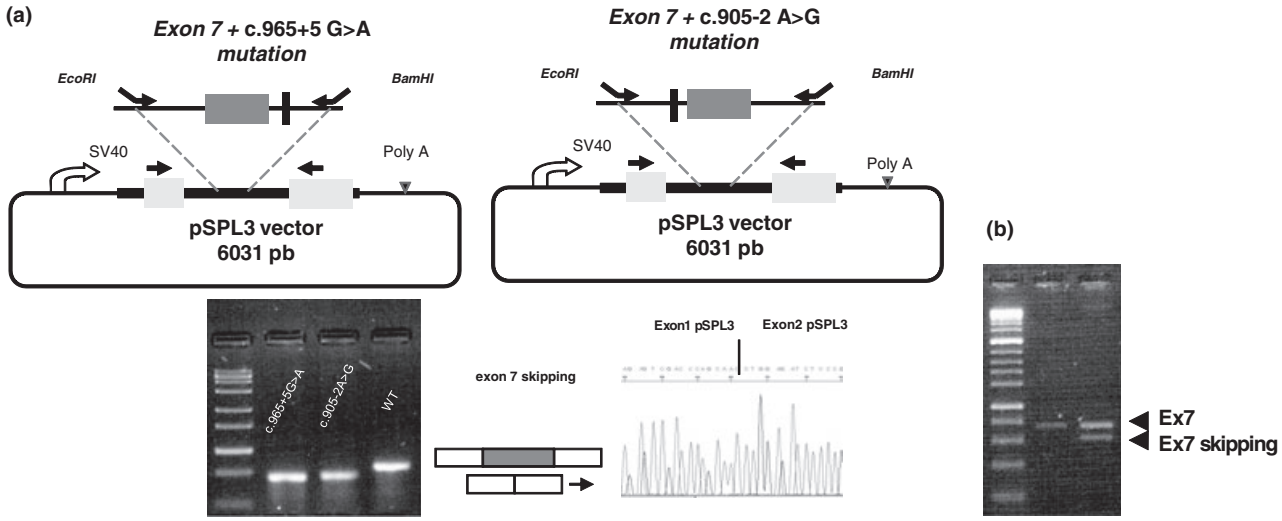

Fig. 2. Splicing assays (**a**). *In vitro* assay using a splicing reporter minigene: Minigene constructions for the two mutations (c.965+5G>A; c.905-2A>G) inducing exon 7 skipping. (**b**) Skipping of exon 7 (60 bp deleted) confirmed by RT-PCR on cDNA from blood sample of patient with c.905-2A>G mutation.

associated with a high relative risk of OC either in a familial or in a sporadic context. In contrast, the relative risk of BC associated with *RAD51C* mutation seems lower since the 0.1% rate of inactivating mutation in a control Finnish population, was not statistically different from the rate observed in BC families. Since submission of this manuscript, Loveday et al. reported a nearly sixfold increased relative risk for OC and the absence of increased risk for BC (15).

Our results are consistent with those of Meindl et al. (6), Romero et al. (10), Pelttari et al. (11), and Thompson et al. (12) since in these studies *RAD51C* mutations were found associated with OC families, but are in contrast to others in which no *RAD51C* mutation was found. These negative results might be explained by stochastic variation because the frequency of *RAD51C* is low. A lack of sensitivity is not probable, but differences in the ascertainment of collected families might explain these differences. In our study, we focused on families and cases with OC cases. We did not find recurrent mutations, as in the study of Pelttari (11) where two recurrent mutations were found in the control population, and within the same haplotype context, showing a founder effect in the Finnish population. In our study, the two splice mutations found are different and were not found in the 12,000 exome variants database (http://genome.sph.umich.edu/wiki/Exome_Chip_Design and ftp://share.sph.umich.edu/exomeChip/IlluminaDesigns/codingContent/), but the stop-gain we found

was observed twice in this non selected population. The ~1:12,000 alleles variant frequency deduced remains compatible with a low frequency risk factor. Other non-synonymous variants are found in this database and in the EVS database (http://evs.gs.washington.edu/EVS/) which is part of the Exome Chip project mentioned above. Among them, the missense variant c.790G>A (p.Gly264Ser) has a 0.28% frequency in Caucasian, close to the 0.24% frequency found in Afro-American subjects. The frequency of this missense variant was not significantly different for any of the patients subgroups compared with control populations in Pelttari's study (11). However, there was a tendency for a higher frequency among OC cases (1%, $p < 0.066$). The recent study of Thompson reported a significant association for this missense variant in OC cases and provides support for the designation of this variant as a moderately penetrant allele (12).

Our experiments show that the two splice mutations are inactivating the gene either by introducing a stop codon or by leading to abnormal splicing, that we documented by the minigene method on the cloned allele.

RAD51C belongs to the homologous recombination DNA repair system, with other genes such as *BRIP1*, *PALB2*, *CHEK2*, *RAD50*, *ATM*, and *BRCA1/2*. Mutations in these genes are associated with increased risk of BC and/or OC. Targeted capture and massively parallel sequencing represents a way to easily test these genes in inherited OC (16). After *RAD51C* was shown to be responsible for OC susceptibility, *RAD51D* mutations were found also in families with ovarian and BC, but no mutation was found in BC only families (3, 4). The increased relative risk for OC compared to BC associated with *RAD51D* mutation, as estimated in the Loveday's study (6.3 *vs* 1.3) (3) likely explains this result, and this is also probably the case for the *RAD51C* gene although this calculation was not possible in our study. The frequency of *RAD51D* deleterious mutations among OC families is close to our results on *RAD51C*, and histological types of OC are also similar. Interestingly, *RAD51D* mutated cells are sensitive *in vitro* to poly(ADP-ribose) polymerase inhibitors, which have been shown to be efficient in BRCA1/2 mutated cells (17). This characteristic might be shared with *RAD51C* cells as they both belong to the same functional class of proteins.

In conclusion, our results confirm that mutations of the *RAD51C* gene are associated with an increased risk of OC, and that a systematic screening of this gene should be included in the search for mutation in ovarian families and cases, since it has consequences both on prevention and potentially on treatment. However, for efficacy purpose only ovarian cases should be tested for *RAD51C* mutation, giving the highest chance for finding a mutation since mutations were only found in this case. Indeed in our study, the *RAD51c* mutation frequency in the ovarian index cases would be higher (3/69). Thus, the list of susceptibility genes for OC includes currently *BRCA1/2, RAD51C, RAD51D*, hereditary non-polyposis cancer genes and other recently identified genes (16). It is likely that the list will further extend beyond those genes and that will increase the screening burden for routine diagnosis and spur the implementation of new generation sequencing for this purpose.

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