The variant E233G of the RAD51D gene could be a low-penetrance allele in high-risk breast cancer families without BRCA1/2 mutations

ARTICLE *in* INTERNATIONAL JOURNAL OF CANCER · JULY 2004

Impact Factor: 5.09 · DOI: 10.1002/ijc.20169 · Source: PubMed

Int. J. Cancer: **110,** 845–849 (2004)

Publication of the International Union Against Cancer

THE VARIANT E233G OF THE *RAD51D* GENE COULD BE A LOW-PENETRANCE ALLELE IN HIGH-RISK BREAST CANCER FAMILIES WITHOUT *BRCA1/2* MUTATIONS

Raquel Rodríguez-López[1]*, Ana Osorio[1], Gloria Ribas[1], Marina Pollán[2], Luis Sánchez-Pulido[3], Miguel de la Hoya[4], Álvaro Ruibal[5], Pilar Zamora[6], Jose Ignacio Arias[7], Raquel Salazar[8], Ana Vega[9], Jose Ignacio Martínez[10], Eva Esteban-Cardeñosa[11], Carmen Alonso[12], Rocío Letón[1], Miguel Urioste Azcorra[1], Cristina Miner[11], M. Eugenia Armengod[7], Angel Carracedo[9], Rogelio González-Sarmiento[8], Trinidad Caldés[4], Orland Díez[13] and Javier Benítez[1]

[1]*Department of Human Genetics, Spanish National Cancer Centre, Madrid, Spain*
[2]*National Epidemiology Centre, Instituto de Salud Carlos III, Madrid, Spain*
[3]*Protein Design Group, National Biotechnology Centre, Madrid, Spain*
[4]*Department of Molecular Oncology, San Carlos Hospital, Madrid, Spain*
[5]*Department of Nuclear Medicine, University Hospital, Santiago de Compostela, Spain*
[6]*Department of Oncology, La Paz Hospital, Madrid, Spain*
[7]*Surgery Service, Monte Naranco Hospital, Oviedo, Spain*
[8]*Department of Internal Medicine, Cancer Research Centre, Salamanca, Spain*
[9]*Department of Molecular Medicine, University Hospital, Santiago de Compostela, Spain*
[10]*Genetics Department, Cytological Research Institute, Valencia, Spain*
[11]*Molecular Biology Department, Faculty of Medicine, University of Valladolid, Valladolid, Spain*
[12]*Department of Oncology, Sant Pau Hospital, Barcelona, Spain*
[13]*Department of Genetics, Sant Pau Hospital, Barcelona, Spain*

Six SNPs have been detected in the DNA repair genes *RAD51C* and *RAD51D*, not previously characterized. The novel variant E233G in *RAD51D* is more highly represented in high-risk, site-specific, familial breast cancer cases that are not associated with the *BRCA1/2* genes, with a frequency of 5.74% ($n = 174$) compared to a control population ($n = 567$) and another subset of breast cancer patients ($n = 765$) with a prevalence of around 2% only (comparison to controls, OR = 2.6, 95% CI 1.12–6.03; $p < 0.021$). We found that the immunohistochemical profile detected in available tumors from these patients differs slightly from those described in non-*BRCA1/2* tumors. Finally, the structural prediction of the putative functional consequence of this change indicates that it can diminish protein stability and structure. This suggests a role for E233G as a low-penetrance susceptibility gene in the specific subgroup of high-risk familial breast cancer cases that are not related to *BRCA1/2*.

Key words: RAD51D; BRCA1/2; *breast cancer*

BRCA1 and *BRCA2* are high-penetrance genes that account for around 25% of families with hereditary breast cancer.[1] These genes have been implicated in the DSB repair pathway by HR, dysfunction of which has been suggested to confer susceptibility to carcinogenesis.[2] Given that no additional high-penetrance susceptibility genes have been found to be involved in breast cancer, it has been proposed that different genetic backgrounds due to the combination of low-penetrance genes (polygenic mechanism) could explain the remaining familial breast cancer risk.[3] In support of the polygenic model, a variant in the gene *CHEK2,* 1100delC mutation, has been described in a population in the north of Europe as the first low-penetrance gene associated with familial breast cancer to confer a 2-fold increase in risk.[4,5] This variant, however, has not been confirmed in our Mediterranean population.[6]

Like *BRCA1/2, CHEK2* is involved in DSB DNA repair, activating *BRCA1* after DNA damage.[7] Other genes involved in DSB DNA repair are good candidate low-penetrance genes that may contribute to the development of breast cancer. In this way, the *RAD51* gene is a eucaryotic homologue of the bacterial RecA, essential to the DSB repair pathway, that interacts with *BRCA1* and *BRCA2*.[8] Many Rad51-related proteins also interact with Rad51 as part of a multiprotein complex that forms a heterodimer with each of the genes playing a different role in DSB repair by HR.[9] They are the *XRCC2, XRCC3, RAD51C* and *RAD51D* genes, some of which (*RAD51, XRCC2* and *XRCC3*) have already been analyzed for genetic variants in breast cancer cases, with some polymorphisms found to confer increased susceptibility.[10,11] The list of such genes, however, is long; and it would be of great interest to increase the number of studies, to clarify the role of this pathway in breast cancer. Thirty-seven genes involved in different DNA repair pathways have been characterized with their cSNPs identified among diverse populations.[12] This constitutes an important catalogue of common variants for molecular epidemiologic studies focusing on individual cancer risk susceptibility. In our study, we genetically characterized 2 new genes of the DNA repair pathway (*RAD51C* and *RAD51D*) not previously included in the catalogue, searching for low-penetrance alleles. We analyzed probands from high-risk familial breast cancer cases, previously excluded because they did not carry mutations in the *BRCA1/2* genes, given that this kind of sample selection simplifies and enhances the power of association studies.[13] Among others, we found a variant in *RAD51D* (E233G) that is significantly more frequent among high-risk, site-specific breast cancer families compared to the control population and other subsets of breast cancer populations. This

Abbreviations: CI, confidence interval; cSNP, single-nucleotide polymorphism; DSB, double-strand; ER, estrogen receptor; HR, homologous recombination; HRF, high-risk family; IDC, infiltrating ductal carcinoma; MIB, Medical Impairment Bureau; OR, odds ratio; PR, progesterone receptor; SSCP, single-strand conformation polymorphism.

Grant sponsor: Fondo de Investigación Sanitaria; Grant numbers: 01/0024, SAF 01/0075, SAF 03/02497; Grant sponsor: Servicio Madrileño de Salud.

*Correspondence to: Department of Human Genetics, Spanish National Cancer Centre, C/Melchor Fernández Almagro 3, 28029 Madrid, Spain. Fax: +34-91-224-69-23. E-mail: rrodriguez@cnio.es

Received 18 September 2003 ; Revised 11 November, 22 December 2003 ; Accepted 5 January 2004

DOI 10.1002/ijc.20169
Published online 24 March 2004 in Wiley InterScience (www.interscience.wiley.com).

could represent the second example of a low-penetrance allele contributing to the molecular basis of breast cancer in a clearly defined number of familial cases.

MATERIAL AND METHODS

Patient selection

Blood samples from index patients of 364 HRF breast cancer families with at least 3 females affected with breast and/or ovarian cancer (one diagnosed before 50 years of age) or at least one male and one female affected were selected (first- and second-degree affected relatives were considered). In each case, the whole coding sequence of the *BRCA1* and *BRCA2* genes had been previously analyzed; 96 cases carried mutations in either the *BRCA1* or *BRCA2* gene and 268 did not. These results have already been published in part.[14] The specific phenotypes of these non-*BRCA1/2* families are shown in Table I. In addition, peripheral blood samples were selected from 629 consecutive and unselected breast cancer patients and 136 from patients who had reported a familial aggregation pattern of breast cancer (one additional case of breast cancer in the family). These samples were collected from various hospitals: the Jiménez Díaz Foundation and La Paz Hospital (Madrid), Monte Naranco Hospital (Oviedo). A control population of 567 women (between 25 and 70 years of age), taken as a representative sample of the genetic background of the Spanish population where cases arose, was used for association studies: 383 individuals from the National Blood Transfusion Center (under the age of 50) and 184 from the traumatology department of the Jiménez Díaz Foundation (over the age of 50). All individuals gave official consent.

PCR amplification

We obtained DNA from peripheral blood lymphocytes of each patient using standard procedures. PCR was performed in 25 μl of a mixture containing 100 ng genomic DNA; 1 × PCR buffer; 200 mM dATP, dTTP, dGTP and dCTP; 25 pmol each primer; 1.75 U Taq polymerase; and 2% DMSO.

The whole coding sequence and exon–intron boundaries of the *RAD51C* and *RAD51D* genes were amplified in 200–400 bp fragments using primers designed by the Primer3 program (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and are available upon request.

Amplification conditions were as follows: exons 1 and 2 of *RAD51C* and exons 1, 3, 7, 8, 9, 10 of *RAD51D* were amplified using 3 cycles of 94°C for 45 sec, 61°C for 30 sec and 72°C for 30 sec; 3 cycles of 94°C for 45 sec, 59°C for 30 sec and 72°C for 30 sec; and 30 cycles of 94°C for 45 sec, 58°C for 40 sec and 72°C for 40 sec. The remaining *RAD51C* and *RAD51D* gene fragments were amplified using the same cycles but with annealing temperatures starting at 60°C, 59°C and 57°C, respectively.

Identification and selection of SNPs

All amplified fragments were fully analyzed in 20 index probands from the HRF breast cancer cases without *BRCA1/2* mutation by direct sequencing using an automated fluorescence-based cycle sequencer (ABI Prism 3700; Perkin-Elmer, Foster City, CA). Detected variants were screened in 96 controls using SSCP to establish allelic frequencies. Selected polymorphisms with a frequency >15% were studied in the first 375 patients from the 629 unselected consecutive cases of breast cancer, with frequency matching to the Spanish control population, using Pyrosequencing 96 (Biotage AB, Westborough, MA). In the rare variant E233G, the TaqMan technique (Applied Biosystems, Foster City, CA) was used to perform wider screening in all cases and controls.

TABLE I – SPECIFIC PHENOTYPE OF THE *BRCA1/2*-NEGATIVE HIGH-RISK FAMILIES

Group of families	Number of families	%
Breast site-specific cancer families[1]		
3 cases or 2 cases with at least one bilateral	100	37.3%
4 cases	42	15.6%
5 cases	25	9.3%
>5 cases	7	2.6%
Total	174	64.8%
Breast and ovarian cancer families	80	29.8%
Male breast cancer families	14	5.4%
All families	268	100%

[1]Refers to familial cases in which the only tumor reported is breast cancer.

Sequence analysis and molecular modeling

Alignment was conducted with the T-Coffee software,[15] viewed with the Belvu program (http://www.cgr.ki.se/cgr/groups/sonnhammer/Belvu.html). The template structures used for RAD51D Swiss homology modeling were 2REB and 1N0W, obtained from the Protein Data Bank (http://www.rcsb.org/pdb/). Structure, rotamers and solvent accessibility were analyzed with WhatIf. Figures were rendered with Insight II (version 98.0; Molecular Simulations).

Morphologic and immunohistochemical studies

A hematoxylin and eosin–stained histologic slide from tumors of each patient was reviewed. The Nottingham histologic grading system was used to assess the grade of IDCs.[16]

Immunohistochemical staining was performed by the LSAB method (Dako, Glostrup, Denmark) with a heat-induced antigen-retrieval step. Sections from the tissue array were immersed in boiling 10 mM sodium citrate (pH 6.5) for 2 min in a pressure cooker. Antibodies, dilutions and suppliers used have been previously published.[17] The percentage of stained nuclei, independent of the intensity, was scored for ER, PR, Ki-67 and p53. Similarly, the percentage of cells with cytoplasmic stain was scored for Bcl2. To evaluate Her2 and cadherins/catenins, percentages of cells with membranous staining and intensity were evaluated. For categorical analysis, a case was considered positive (cut off) when at least 10%, 10%, 25% and 70% of the cells were stained with ER, PR, p53 and Bcl2, respectively.[18] Three categories were defined for Ki-67: 0–5%, 6–25% and >25% of stained nuclei. Her2 was evaluated according to the 4-category (0–3+) Dako system proposed for evaluation of the HercepTest.

Statistical analysis

Genotypic frequencies in each set of cases and controls were computed, with Hardy-Weinberg equilibrium testing. The distribution of the E233G variant among controls was compared to the frequencies in sporadic and familial cases using χ^2 tests. Regarding familial cases, a distinction was made according to *BRCA1/2* status. Furthermore, *BRCA1/2*-negative patients with a familial history of breast cancer as the only tumor among their relatives were singled out as a specific subgroup. Estimated risks for each selected polymorphism were calculated as ORs with associated 95% CIs.

Fisher's exact test was used to compare the observed immunohistochemical characteristics (in percentages) of the 5 E233G-positive tumors to the 28 negative ones.

RESULTS

Sequence variations in the *RAD51C* and *RAD51D* genes were analyzed in the index cases of 20 high-risk families. To this end, we directly sequenced the whole coding region and exon–intron boundaries of *RAD51C* and *RAD51D* (8 and 10 exons, respectively). We found 2 SNPs in *RAD51C* and 4 in *RAD51D.* All germline variants detected along with their allelic frequencies in 96 control individuals are summarized in Table II. Five of the 6 variants were directly classified as polymorphisms because their heterozygous frequencies were higher than 1% in the control population and/or they were silent changes. The E233G variant in the *RAD51D* gene was the only one that could not be automatically

classified as a polymorphism as it resulted in an interesting amino acid change and demonstrated a low frequency of heterozygotes in the 567 controls studied.

Case-control studies were carried out for the 2 common SNPs that had an allelic frequency near or higher than 15% in the control group: IVS6+34pb T/C (28%) in the *RAD51C* gene and R126Q (14%) in *RAD51D* (Table II). Genotype frequencies were compared to a set of 375 consecutive breast cancer cases and 388 frequency age-matched controls (data not shown). No differences were reported between the control and breast cancer populations. Relative risks for homozygosity were as follows: for the IVS6+34pb polymorphism, OR = 1.15, 95% CI 0.75–1.76, and for R126Q, OR = 1.04, 95% CI 0.33–3.27. No deviations from Hardy-Weinberg equilibrium were observed.

In relation to the E233G variant of the *RAD51D* gene that we found at a low frequency among the whole control population, we investigated its possible significance by extending analysis to a total of 765 patients with breast cancer; 629 of them were consecutive and 136 selected for presenting one additional breast cancer case in the family (familial aggregation pattern). No homozygosity was found; the variant appeared heterozygous with a frequency of 2.29% among the control population, and prevalences in the consecutive patients and those with a familial aggregation pattern were 2.38% and 1.47%, respectively (comparison to controls $p = 0.916$ and $p = 0.551$) (Table III).

We then studied a group of probands from 364 high-risk breast cancer families previously screened for the *BRCA1/2* genes: 96 cases carried mutations in one of the 2 genes and showed a prevalence of 3.12% for the E233G variant (comparison to controls $p = 0.623$). Among the 268 cases not associated with the *BRCA1/2* genes, however, the prevalence of heterozygosity for the variant increased slightly to 3.73%, though it did not demonstrate statistical significance (OR = 1.65, 95% CI 0.71–3.82). We investigated the clinical characteristics of these cases and found that all cases carrying the variant belonged to site-specific breast cancer families. The prevalence of heterozygosity for the variant among site-specific breast cancer families ($n = 174$) increased to 5.74% and differed significantly from that observed in the control group (OR = 2.60, 95% CI 1.12–6.03; $p < 0.021$) (Table III).

To test this association, we performed a segregation analysis in 2 cases carrying the variant for which we had DNA from additional members of the families, but the analysis showed that the E233G variant did not totally segregate with the disease (data not shown).

To analyze the nature of these E233G-positive cases in greater depth, we investigated tumor characteristics. In 5 of the 10 familial cases positive for the E233G variant, the morphology and immunochemistry of the tumors were available (Table IV); we observed that, in contrast to *BRCA1/2*-negative tumors,[18] the majority of tumors positive for E233G were grade 2/3 (80%), ER- and PR-negative (approx. 60%) and Bcl2-negative (80%) and had higher

TABLE II – GERMLINE VARIANTS FOUND IN THE RAD51C AND RAD51D GENES

	Change	Designation	Allelic frequency[1]	Significance
RAD51C gene (NM_002876)[2]				
Exon 1	G/A	A30A	0.5%	SNP
Exon 6	T/C	IVS6 + 34pb	28%	SNP
RAD51D gene (NM_133630)				
Exon 3	C/T	S78S	5.7%	SNP
Exon 6	G/A	R126Q	14%	SNP
Exon 8	A/G	E233G	1.1%[3]	Unknown
3-UTR	G/A	107pb	1%	SNP

[1]Allelic frequencies are given for a control population of 96 individuals.– [2]NM, locus designation from HUGO Gene Nomenclature Committee guidelines.– [3]The number of individuals studied for this polymorphism was 567.

TABLE III – PREVALENCE OF PEOPLE WITH THE E233G VARIANT IN THE DIFFERENT STUDIED GROUPS

Population	Number of individuals	E233G carrier	Prevalence of heterozygosity[1] (95% CI)	*p* VALUES[2]
Controls (total)	567	13	2.29% (1.23–3.89)	
Women <50 years	383	9	2.34% (1.10–4.41)	
Women >50 years	184	4	2.17% (0.60–5.47)	
Consecutive cases	629	15	2.38% (1.34–3.90)	0.916
Aggregation families	136	2	1.47% (0.18–5.21)	0.551
High-risk families	364	13	3.57% (1.92–6.03)	0.248
BRCA-positives	96	3	3.13% (0.65–8.86)	0.623
BRCA-negatives	268	10	3.73% (1.80–6.75)	0.236
BRCA-negatives only with breast cancer cases	174	10	5.74% (2.79–10.31)	0.021

[1]Proportion of people carring the variant. Since no homozygous cases were found, the allelic frequency is just half of the presented percentage.– [2]χ^2 square test, comparisons against the control population as a whole.

TABLE IV – DIFFERENTIAL CLINICAL AND IMMUNOHISTOCHEMICAL CHARACTERISTICS OF E233G-POSITIVE TUMORS BELONGING TO HRF BREAST CANCER CASES

	Grade	ER (10%)[1]	PR (10%)	Bcl-2 (70%)	p53 (25%)	MIB (5%)	cErb2
BRCA1/2^{-}[2] (% of tumors)	1 (50%)	+ (75%)	+ (68%)	+ (50%)	− (96%)	− (92%)	0/+ (96%)
F.57SP	1	+	−	−	−	+	++
F.68CNIO	2	+	+	+	+	+	0
F.141HCSC	3	−	+	−	−	+	+++
F.146CNIO	2	−	−	−		+	
F.150CNIO	2	−	−	−	−	+	++
Summary[3]	(20%)	(40%)	(40%)	(20%)	(75%)	(0%)	(25%)
p value[4]	0.346	0.149	0.328	0.346	0.284	<0.001	0.003

[1]Number in parentheses for ER, PR, Bcl2, p53 and MBI refers to the cut-off for defining positivity or negativity.– [2]Refers to familial tumors without *BRCA1/2* mutation. These data correspond to 28 cases that we studied from a morphologic and immunohistochemical point of view.[18] Number in parentheses refers to the percentage of cases with this characteristic.– [3]Summary of the characteristics of E233G positive and *BRCA1/2*$^{-}$ tumors. [4]Fisher's exact test), comparing the observed percentages between the 5 E233G-positive cases and the 28 negative ones.

MIB values than expected (100%). The percentage of c-erbB-2-negative tumors also decreased in this group.

Finally, to investigate the possible functional consequence of this change, we studied the substitution of glutamic acid-233 for glycine using structural modeling of mutant Rad51D containing the variant (Fig. 1, upper). Although Rad51D is not crystallized, its suitable homology to the bacterial RecA protein[9] allowed us to predict the consequences of the amino acid change. Our model showed that the change to glycine in the 233 position could affect the Rad51D structure in at least 2 different ways. Firstly, the glutamic acid in the 233 conserved position is located at the middle of helix F, whose replacement for glycine diminishes its stability.[19] Secondly, glutamic acid-233 (K177 in RecA) is a candidate for establishing an electrostatic interaction with arginine-186 (D130 in RecA), mimicking the semiburied salt bridge found at the corresponding positions in the RecA structure, between aspartic acid-130 and lysine-177. Substitution of glutamic acid-233 with glycine eliminates this hypothetical salt bridge, which is conserved through the phylogenetic scale in RecA, and may diminish the protein stability and affect the Rad51D structure (Fig. 1, lower).[20]

DISCUSSION

A catalogue of SNPs with 37 genes of DNA repair pathways has been published.[12] This constitutes an important tool for association studies of human disease, specifically in relation to cancer because many individuals exhibit a reduced repair capacity that can be associated with cancer risk.

The *RAD51C* and *RAD51D* genes are involved in the same pathway, but they were not included in this list or previously analyzed from a genetic point of view. In this context, we studied the coding sequence and exon–intron boundaries of both genes to detect common polymorphisms and/or susceptibility alleles that could explain the appearance of the disease.

We detected 6 germline genetic alterations, 2 of them cSNPs, in the 20 probands. Five were directly considered to be polymorphisms because either they had a frequency of heterozygosity >1% in the control population or they were silent changes in all but 2 cases. IVS6+34pbT/C in *RAD51C* and R126Q in *RAD51D* presented frequencies near or higher than 15% and were suitable for association testing with breast cancer susceptibility in a case-control study. When comparing the allelic frequencies between the 329 nonfamilial breast cancer cases and their paired controls, however, no statistically significant differences were found for either of the 2 polymorphisms. We concluded that none of these frequent polymorphisms demonstrated any tendency toward breast cancer risk association.

Concerning the E233G variant of the *RAD51D* gene that we found in a low frequency among the control population (567 individuals), we investigated its possible significance by extending the analysis to a total of 765 patients (629 consecutive and 136 with familial aggregation pattern) and 364 high-risk breast cancer patients The variant appeared at a similar frequency among the different groups (2.29% controls, 2.38% consecutive patients, 1.47% familial aggregation and 3.57% high-risk patients) (Table III). We investigated the clinical characteristics of the high-risk group and found that all cases carrying the variant belonged to site-specific breast cancer families (*BRCA1/2*-negative). The prevalence of the variant in this group of site-specific breast cancer families was significantly higher than in controls (OR = 2.60, 95% CI 1.12–6.03; $p < 0.021$) (Table III).

Given the low prevalence of this new variant, our study lacked sufficient statistical power to detect a relative risk in the range expected for low-penetrance alleles. Indeed, the number of controls and cases would have allowed detection only of excesses >2-fold. The higher prevalence of the E233G variant in cases from high-risk families that had no mutations in *BRCA1* or *-2* and reported breast cancer only deserves further study.

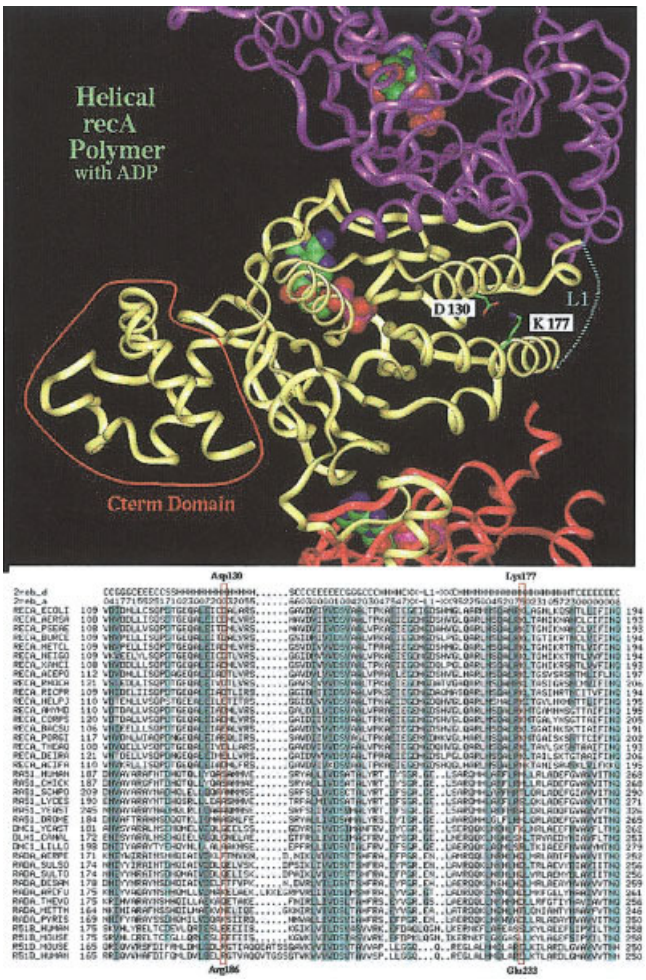

FIGURE 1 – Salt bridge in RecA and Rad51D model. *(Upper)* Salt bridge in the RecA structure that brings 2 α-helixes nearer, located just above the DNA domain of protein interaction. Image corresponds to the predicted salt bridge for the Rad51D model between glutamic acid-233 (K177 in RecA) and arginine-186 (D130 in RecA), which is destabilized for the glycine-233 mutant model. *(Lower)* Alignment among representative members of the RecA/RAD51 family on a phylogenetic scale. Numbers preceding and following the alignment indicate the positions of the first and last residues in each sequence. The secondary structure and accessibility for 2REB (RecA from *Escherichia coli*) are shown above the alignment. Alignment viewed with Belvu program (see Material and Methods section) indicate average BLOSUM62 score in each column correlated to amino acid conservation from higher to lower values: cyan, >3; light red, 1.5–3.0; light green, 0.5–1.5. Sequences are named using the SWISSPROT identifications (http://www.ebi.ac.uk/swissprot/).

To test this association, we performed a segregation analysis in 2 cases carrying the variant for which we had DNA from additional family members, but the analysis showed that the E233G variant did not totally segregate with the disease (data not shown). Although the lack of segregation within these families could suggest that E233G is a polymorphism, such incomplete segregation has been described for the breast cancer susceptibility variant 1100delC in the *CHEK2* gene, which is also more frequent among patients with familial breast cancer not associated with *BRCA1/2* mutations.[4] This kind of segregation was explained by the role of the 1100delC variant as a low-susceptibility allele not solely responsible for the breast cancer susceptibility in these families. The same mechanism could be applied to the E233G variant in our population and could represent the second example of a low-penetrance breast cancer gene involved in these families. As in the

case of 1100delC, E233G could be responsible for a percentage of the risk observed in these high-risk families. Other predisposition genes are necessary to explain the clustering of the disease, as previously reported by others.[4,21]

Unfortunately, the 1100delC variant of *CHEK2* was not present in our population.[6]

To analyze the nature of these E233G-positive cases in greater depth, we investigated tumor characteristics. We previously reported that familial tumors not associated with *BRCA1/2* mutations have specific immunohistochemical characteristics compared to *BRCA1*- and *BRCA2*-positive tumors.[17] Non-*BRCA1/2* tumors are frequently grade 1 (50%), ER- and PR-positive (75% and 68%, respectively) and Bcl2-positive (50%) and have very low proliferation (MIB) and p53 values (<25%) in >90% of cases. In 5 of the 10 familial cases positive for the E233G variant, the morphology and immunochemistry of the tumors were available (Table IV); the majority of E233G-positive tumors were grade 2/3 (80%), ER- and PR-negative (60%) and Bcl2-negative (80%) and had higher MIB values than expected (100% *vs.* 8% in non-*BRCA1/2* tumors). Our results therefore suggest that E233G could contribute to a fraction of familial breast cancer cases whose tumors appear to present a different and less favorable clinical pattern, accounting for a minority subset among the group of non-*BRCA1*/2 tumors. Although the number of cases is too small to extract any conclusions, this preliminary observation can contribute to the future delimitation of these subgroups by comparing the same markers.

Finally, to investigate the possible functional consequence of this change, we studied the substitution of glutamic acid-233 for glycine using structural modeling of the mutant Rad51D containing the variant (Fig. 1, upper). Our model using the homologous RecA protein[9] showed that the change to glycine in the 233 position could affect the Rad51D structure in at least 2 different ways, diminishing its stability and eliminating the hypothetical salt bridge that establishes an electrostatic interaction between glutamic acid-233 (K177 in RecA) with arginine-186 (D130 in RecA) and that is conserved through the phylogenetic scale in RecA (Fig. 1, lower).[20]

In summary, we identified 6 variants in the *RAD51C* and *RAD51D* genes. Five of them do not appear to be associated with breast cancer risk, but the E233G variant could be overrepresented among site-specific, high-risk breast cancer families (OR = 2.60, 95%CI 1.12–6.03). Although there are few available tumors from E233G carriers, they do demonstrate immunohistochemical characteristics that differ slightly from those described for *BRCA1/2*-negative tumors and could constitute a more aggressive signature of a small fraction of these patients. Finally, the prediction of this change suggests that it can diminish protein stability and structure.

Due to the fact that our observation about the E233G variant depends on a particular subgroup of non-*BRCA1/2* familial breast cancer cases, our findings need to be confirmed in other populations. The E233G variant in the *RAD51D* gene could constitute the second low-penetrance breast cancer susceptibility allele. Analysis of populations in which 1100delC in *CHEK2* and E233G in *RAD51D* are prevalent would be of extreme interest in determining their possible interaction in a breast cancer susceptibility polygenic model.

ACKNOWLEDGEMENTS

We thank Dr. A. Vicario from the Regional Transfusion Center (Madrid) and Dr. Jm San Román from the Jiménez Diaz Foundation (Madrid) for providing the DNA control samples and patients. We also thank Ms. R. Letón for technical assistance and Ms. A. Wren for reviewing the manuscript. R.R.-L. is a fellow of Fondo de Investigación Sanitaria and A.O. is from Comunidad de Madrid.

REFERENCES

1. Ford D, Easton DF, Stratton M, Narod S, Goldgar D, Devilee P, Bishop DT, Weber B, Lenoir G, Chang-Claude J, Sobol H, Teare MD, et al. Genetic heterogeneity and penetrance analysis of the *BRCA1* and *BRCA2* genes in breast cancer families. Breast Cancer Linkage Consortium. Am J Hum Genet 1998;62:676–89.
2. Scully R, Livingston DM. In search of the tumor-suppressor functions of *BRCA1* and *BRCA2*. Nature 2000;408:429–32.
3. Pharoah PD, Antoniou A, Bobrow M, Zimmern RL, Easton DF, Ponder BA. Polygenic susceptibility to breast cancer and implications for prevention. Nat Genet 2002;31:33–6.
4. 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, Elstrodt F, van Duijn C, et al. Low-penetrance susceptibility to breast cancer due to CHEK2(*)1100delC in noncarriers of *BRCA1* or *BRCA2* mutations. Nat Genet 2002;31:55–9.
5. Vahteristo P, Bartkova J, Eerola H, Syrjakoski K, Ojala S, Kilpivaara O, Tamminen A, Kononen J, Aittomaki K, Heikkila P, Holli K, Blomqvist C, et al. A *CHEK2* genetic variant contributing to a substantial fraction of familial breast cancer. Am J Hum Genet 2002; 71:432–8.
6. Osorio A, Rodríguez-López R, Diez O, de la Hoya M, Martinez JI, Vega A, Esteban-Cardeñosa E, Alonso C, Carracedo A, Caldes T, Benítez J. The breast cancer low-penetrance allele 1100delC in the *CHEK2* gene is not present in Spanish familial breast cancer population. Int J Cancer 2004;108:54–6.
7. Lee JS, Collins KM, Brown AL, Lee CH, Chung JH. hCds1-mediated phosphorylation of *BRCA1* regulates the DNA damage response. Nature 2000;404:201–4.
8. Davies AA, Masson JY, McIlwraith MJ, Stasiak AZ, Stasiak A, Venkitaraman AR, West SC. Role of *BRCA2* in control of the RAD51 recombination and DNA repair protein. Mol Cell 2001;7:273–82.
9. Schild D, Lio YC, Collins DW, Tsomondo T, Chen DJ. Evidence for simultaneous protein interactions between human Rad51 paralogs. J Biol Chem 2000;275:16443–9.
10. Kuschel B, Auranen A, McBride S, Novik KL, Antoniou A, Lipscombe JM, Day NE, Easton DF, Ponder BA, Pharoah PD, Dunning A. Variants in DNA double-strand break repair genes and breast cancer susceptibility. Hum Mol Genet 2002;11:1399–407.
11. Wang WW, Spurdle AB, Kolachana P, Bove B, Modan B, Ebbers SM, Suthers G, Tucker MA, Kaufman DJ, Doody MM, Tarone RE, Daly M, et al. A single nucleotide polymorphism in the 5′ untranslated region of RAD51 and risk of cancer among *BRCA1/2* mutation carriers. Cancer Epidemiol Biomarkers Prev 2001;10:955–60.
12. Mohrenweiser HW, Xi T, Vazquez-Matias J, Jones IM. Identification of 127 amino acid substitution variants in screening 37 DNA repair genes in humans. Cancer Epidemiol Biomarkers Prev 2002;11:1054–64.
13. Houlston RS, Peto J. The future of association studies of common cancers. Hum Genet 2003;112:434–5.
14. Díaz O, Osorio A, Durán M, Martínez-Ferrandis JI, de la Hoya M, Salazar R, Vega A, Campos B, Rodríguez-López R, Vlasco E, Chaves J, Díaz-Rubio E, et al. 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 2003; 22:301–12.
15. Notredame C, Higgins DG, Heringa J. T-Coffee: a novel method for fast and accurate multiple sequence alignment. J Mol Biol 2000;302: 205–17.
16. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991;19:403–10.
17. Palacios J, Honrado E, Osorio A, Cazorla A, Sarrio D, Barroso A, Rodriguez S, Cigudosa JC, Diez O, Lerma E, Rivas C, Benitez J. Immunohistochemical characteristics defined by tissue microarray of hereditary breast cancer not attributable to *BRCA1* and *BRCA2* mutations: differences with breast carcinomas arising in *BRCA1* and *BRCA2* mutation carriers. Clin Cancer Res 2003;9:3606–14.
18. Torhorst J, Bucher C, Kononen J, Haas P, Zuber M, Kochli OR, Mross F, Dieterich H, Moch H, Mihatsch M, Kallioniemi OP, Sauter G. Tissue microarrays for rapid linking of molecular changes to clinical endpoints. Am J Pathol 2001;159:2249–56.
19. Kumar S, Bansal M. Dissecting alpha-helices: position-specific analysis of alpha-helices in globular proteins. Proteins 1998;31:460–76.
20. Dong F, Zhou HX. Electrostatic contributions to T4 lysozyme stability: solvent-exposed charges versus semi-buried salt bridges. Biophys J 2002;83:1341–7.
21. Antoniou AC, Easton DF. Polygenic inheritance of breast cancer: implications for design of association studies. Genet Epidemiol 2003; 25:190–202.