Published in final edited form as:
Nat Genet. ; 43(9): 879–882. doi:10.1038/ng.893.

Germline mutations in *RAD51D* confer susceptibility to ovarian cancer

Chey Loveday[#1], **Clare Turnbull**[#1], **Emma Ramsay**[1], **Deborah Hughes**[1], **Elise Ruark**[1], **Jessica R Frankum**[2], **Georgina Bowden**[1], **Bolot Kalmyrzaev**[1], **Margaret Warren-Perry**[1], **Katie Snape**[1], **Julian W Adlard**[3], **Julian Barwell**[4], **Jonathan Berg**[5], **Angela F Brady**[6], **Carole Brewer**[7], **Glen Brice**[8], **Cyril Chapman**[9], **Jackie Cook**[10], **Rosemarie Davidson**[11], **Alan Donaldson**[12], **Fiona Douglas**[13], **Lynn Greenhalgh**[14], **Alex Henderson**[15], **Louise Izatt**[16], **Ajith Kumar**[17], **Fiona Lalloo**[18], **Zosia Miedzybrodzka**[19], **Patrick J Morrison**[20], **Joan Paterson**[21], **Mary Porteous**[22], **Mark T. Rogers**[23], **Susan Shanley**[24], **Lisa Walker**[25], **Breast Cancer Susceptibility Collaboration (UK)**[26], **Diana Eccles**[27], **D. Gareth Evans**[28], **Anthony Renwick**[1], **Sheila Seal**[1], **Christopher J Lord**[2], **Alan Ashworth**[2], **Jorge S Reis-Filho**[2], **Antonis C Antoniou**[29], and **Nazneen Rahman**[1]

[1]Section of Cancer Genetics, The Institute of Cancer Research, Sutton

[2]The Breakthrough Breast Cancer Research Centre, The Institute of Cancer Research, Fulham Road, London

[3]Yorkshire Regional Centre for Cancer Treatment, Cookridge Hospital, Leeds

[4]Leicestershire Genetics Centre, University Hospitals of Leicester NHS Trust

[5]Human genetics, Division of Medical Sciences, University of Dundee

[6]NW Thames Regional Genetics Service, Kennedy Galton Centre, London

[7]Peninsula Regional Genetics Service, Royal Devon & Exeter Hospital, Exeter

[8]SW Thames Regional Genetics Service, St George's Hospital, London

[9]West Midlands Regional Genetics Service, Birmingham Women's Hospital, Birmingham

[10]Sheffield Regional Genetics Service, Sheffield Children's NHS Foundation Trust

[11]West of Scotland Regional Genetics Service, FergusonSmith Centre for Clinical Genetics, Glasgow

[12]South Western Regional Genetics Service, University Hospitals of Bristol NHS Foundation Trust

Correspondence should be addressed to N.R. (nazneen.rahman@icr.ac.uk).
[26]A full list of members appears in the Supplementary Note

Author contributions

N.R., C.L. and C.T. designed the experiment. M.W.P., C.T. and N.R. coordinated recruitment to FBCS. J.W.A., J.Ba, J.Be., A.F.B., C.B., G.Br., C.C., J.C., R.D., A.D., F.D., D.G.E., D.E., L.G., A.H., L.I., A.K., F.L., Z.M., P.J.M., J.P., M.P., M.T.R., S.Sh. and L.W. coordinated FBCS sample recruitment from their respective Genetics centres. C.L., E.Ra., D.H., G.Bo., B.K., K.S, A.R. and S.Se. performed sequencing of *RAD51D*. J.R.F., C.J.L. and A.A. designed and conducted drug sensitivity experiments. J.S.R-F. undertook examination and dissection of pathological specimens. C.T., E.Ru. and A.C.A. performed statistical analyses. C.L., C.T. and N.R. drafted the manuscript with substantial input from D.G.E., D.E., A.C.A., A.A., and J.S.R-F. C.T. and N.R. oversaw and managed all aspects of the study.

Accession numbers

RAD51D mutation nomenclature corresponds to Ensembl Transcript ID ENST00000345365.

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[13]Northern Genetics Service, Newcastle upon Tyne Hospitals NHS Foundation Trust

[14]Cheshire and Merseyside Clinical Genetics Service, Alder Hey Children's NHS Foundation Trust, Liverpool

[15]Northern Genetics Service (Cumbria), Newcastle upon Tyne Hospitals NHS Trust

[16]SE Thames Regional Genetics Service, Guy's and St Thomas NHS Foundation Trust

[17]NE Thames Regional Genetics Service, Great Ormond St Hospital, London

[18]University Dept of Medical Genetics & Regional Genetics Service, St Mary's Hospital, Manchester

[19]University of Aberdeen and North of Scotland Clinical Genetics Service, Aberdeen Royal Infirmary

[20]Northern Ireland Regional Genetics Service, Belfast HSC Trust, & Department of Medical Genetics, Queen's University Belfast

[21]East Anglian Regional Genetics Service, Cambridge University Hospitals NHS Foundation Trust

[22]South East of Scotland Clinical Genetics Service, Western General Hospital, Edinburgh

[23]All Wales Medical Genetics Service, University Hospital of Wales, Cardiff

[24]Royal Marsden NHS Foundation Trust, Royal Marsden NHS Foundation Trust

[25]Oxford Regional Genetics Service, Oxford Radcliffe Hospitals NHS Trust

[27]Faculty of Medicine, University of Southampton, Southampton University Hospitals NHS Trust

[28]University Dept of Medical Genetics & Regional Genetics Service, St Mary's Hospital, Manchester

[29]Center for Cancer Genetic Epidemiology, Department of Public Health and Primary Care, University of Cambridge

[#] These authors contributed equally to this work.

Abstract

Recently *RAD51C* mutations were identified in families with breast and ovarian cancer[1]. This observation prompted us to investigate the role of *RAD51D* in cancer susceptibility. We identified eight inactivating *RAD51D* mutations in unrelated individuals from 911 breast-ovarian cancer families compared with one in 1060 controls (P=0.01). The association was principally with ovarian cancer with three mutations identified in the 59 pedigrees with three or more ovarian cancer cases (P=0.0005). The relative risk of ovarian cancer for *RAD51D* mutation carriers was estimated to be 6.30 (95%CI: 2.86-13.85; $P=4.8\times10^{-6}$). By contrast, the relative risk of breast cancer was estimated to be 1.32 (95%CI: 0.59-2.96; P=0.50). These data indicate that *RAD51D* mutation testing may have clinical utility in individuals with ovarian cancer and their families. Moreover, we show that cells deficient in RAD51D are sensitive to treatment with a PARP inhibitor, suggesting a possible therapeutic approach for cancers arising in *RAD51D* mutation carriers.

Homologous recombination (HR) is a mechanism for repairing stalled replication forks, DNA interstrand crosslinks and double-strand breaks[2]. Constitutional inactivating mutations in several genes that encode proteins crucial for DNA repair by HR have been shown to predispose to cancer[3]. In particular, there is a strong association with female cancers and mutations in genes such as *BRCA1, BRCA2, ATM, BRIP1, CHEK2, PALB2, RAD50* and *RAD51C* have been shown to confer susceptibility to breast and/or ovarian cancer[1,4]. Indeed, the analysis of families with breast and ovarian cancer was crucial to the mapping of the *BRCA1* gene[5]. For many years, it was widely believed that the genetic contribution to families with breast and ovarian cancer was largely attributable to mutations in *BRCA1* and *BRCA2*[6-8]. However, last year Meindl *et al.* identified mutations in *RAD51C* in breast-ovarian cancer families[1]. This suggested that analysis of such families may still have utility in cancer predisposition gene discovery.

In eukaryotic cells, DNA repair by HR involves several proteins of which a central player is the DNA recombinase RAD51, the ortholog of bacterial RecA[9]. RAD51 forms helical filaments on DNA and catalyzes DNA strand invasion and exchange. Multiple other proteins are involved in these processes including five RAD51 paralogs: RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3[10]. Here, through a case-control mutation study, we demonstrate that mutations in *RAD51D* (also known as *RAD51L3*) predispose to cancer in humans.

We sequenced the full coding sequence and intron-exon boundaries of *RAD51D* in DNA from unrelated probands from 911 breast-ovarian cancer families and 1060 population controls (Supplementary Table 1). The breast-ovarian cancer families included at least one case of breast cancer and at least one case of ovarian cancer and all were negative for mutations in *BRCA1* and *BRCA2* (Supplementary Table 2).

We identified inactivating mutations in *RAD51D* in eight of 911 cases and one of 1060 controls (P=0.01) (Table 1). The mutations were not equally distributed within the series, with a higher prevalence in families with more than one ovarian cancer; four mutations were detected in 235 families with two or more cases of ovarian cancer (P=0.005) and three mutations were detected in the 59 families with three or more cases of ovarian cancer (P=0.0005) (Fig. 1).

All the mutations are predicted to result in protein truncation through frameshifting insertions or deletions (n=3), the generation of nonsense codons (n=4) or splice defects (n=2) (Table 1).We also identified 5 intronic, 3 synonymous and 15 non-synonymous variants. Three coding variants, rs9901455 (S78S), rs4796033 (R165Q) and rs28363284 (E233G) have minor allele frequency >1% and no association was observed for any of these variants (Supplementary Table 3). Of the remaining rare variants, three were present in both cases and controls, nine were detected in a single case and eight were detected in a single control (Supplementary Table 4). There was thus no overall difference in the frequency of non-truncating *RAD51D* variants between cases and controls. Moreover, there was no difference in the position or predicted functional effects of these variants and it is noteworthy that an equal number (n=5) of non-synonymous variants detected in cases and controls are predicted to affect function (Supplementary Fig. 1 and Supplementary Table 4).

These data indicate that mutations that result in inactivation of RAD51D function predispose to cancer, but that variants with less significant functional effects are likely to be non-pathogenic.

We tested for the family mutation in samples from 13 relatives. This revealed that five of five individuals affected with ovarian or breast cancer carried the family mutation, whereas six of eight unaffected relatives did not carry the family mutation. Several other cancers were present in relatives, such as pancreatic, prostate and colorectal cancer (Fig. 1). However, the mutation status of these individuals is not known and additional studies will be required to evaluate whether *RAD51D* mutations predispose to other cancers. Pathology information was available for four ovarian cancers from *RAD51D* mutation carriers; three were serous adenocarcinoma and one was an endometrioid cancer. Pathology information was available for eight breast cancers of which seven were ductal in origin and one was a carcinoma with medullary features. Receptor status was available from five breast cancers of which three were estrogen receptor positive and two were negative. Tumor material was available from two ovarian cancers and two breast cancers. We detected loss of the wild-type allele in one ovarian and one breast cancer and reduction of the proportion of the wild-type allele in a further breast cancer. In the final ovarian cancer the mutant allele was lost and the wildtype allele was retained (Table 1 and Supplementary Fig. 2).

These characteristics are typical of the intermediate-penetrance cancer predisposition genes that we, and others, have described in breast cancer[1,4,11-14]. To estimate directly the risks associated with *RAD51D* mutations we undertook modified segregation analysis, by modelling the risks of ovarian and breast cancer simultaneously and incorporating the information from the controls and full pedigrees of both mutation-positive and mutation-negative breast-ovarian cancer families. The ovarian cancer relative risk for *RAD51D* mutation carriers was estimated to be 6.30 (95%CI: 2.86-13.85; $P=4.8\times10^{-6}$) (Fig.2). By contrast, the association with breast cancer risk was not statistically significant (RR= 1.32 (95%CI: 0.59-2.96; $P=0.50$).

To further explore the role of *RAD51D* mutations in breast cancer predisposition, we sequenced the gene in an additional series of 737 unrelated individuals from pedigrees in which there was familial breast cancer but no ovarian cancer. We did not identify any inactivating mutations (0/737 cases vs 1/1060 controls $P=1.0$). Although at first glance these data may seem surprising, they are consistent with the results of the segregation analysis. This is because if *RAD51D* mutations confer a sizeable relative risk of ovarian cancer but only a small, or no, increase in breast cancer risk, the frequency of *RAD51D* mutations in a series of breast cancer families selected on the basis of *not* containing ovarian cancer would be anticipated to be very low. The data are also consistent with the detection of *RAD51D* mutations in seven individuals with breast cancer in the breast-ovarian cancer families, as we specifically ascertained the ovarian cancer cases because of their close family history of breast cancer. This will inevitably result in an enrichment of breast cancer in relatives of *RAD51D* mutation-positive ovarian cancer cases, irrespective of whether such mutations confer a risk of breast cancer. To formally refine the risk of breast cancer associated with *RAD51D* mutations will likely be very challenging because the population frequency of *RAD51D* mutations is so low. Assuming a population mutation frequency of 0.1% and a

relative risk of breast cancer of 1.3, full gene mutational analysis of *RAD51D* in 275,000 cases and 275,000 controls would be required to have 90% power to demonstrate the association.

Our data clearly demonstrate that *RAD51D* is an ovarian cancer predisposition gene but further studies in familial and sporadic ovarian cancer series would be of value to further clarify the risks of ovarian cancer. *RAD51D* mutation analysis in individuals with Fanconi anemia and Fanconi-like disorders would also be of interest, given that biallelic mutations in *BRCA2, PALB2, BRIP1* and *RAD51C* have been demonstrated to cause these phenotypes[15-18].

Our discovery has potential clinical utility both for individuals with cancer and their relatives. Cancer patients with *RAD51D* mutations may benefit from specific therapies such as Poly (ADP-Ribose) Polymerase (PARP) inhibitors, which have shown efficacy in patients with impairment of HR due to mutations in *BRCA1* or *BRCA2*[19]. To investigate this we used RNA interference (RNAi) and assessed the relationship between *RAD51D* loss of function and the sensitivity of tumor cells to a clinical PARP inhibitor, olaparib (AstraZeneca). Short interfering (si) RNAi reagents targeting *RAD51D* caused olaparib sensitivity of a magnitude similar to that achieved using silencing of *BRCA2* (Fig. 3a,b), an observation in keeping with the HR defect observed in *RAD51D* null rodent cell lines[20]. To extend this analysis, we also observed the *RAD51D* selective effect of olaparib in *RAD51D* deficient CHO cells in which both alleles of RAD51D have been rendered dysfunctional by gene targeting (Fig. 3c)[20]. These data suggest that PARP inhibitors may have clinical utility in individuals with *RAD51D* mutations. We estimate that only ~0.6% of unselected individuals with ovarian cancer will harbour *RAD51D* mutations, but as we enter an era in which genetic testing will become routine, such individuals will be readily identifiable. Their identification will also be of potential value to female relatives, as those with mutations will be on average at ~6 fold increased risk of ovarian cancer, which equates to an ~10% cumulative risk by age 80. An appreciable proportion of women at this level of risk may consider strategies such as laprascopic oophorectomy, which is well-tolerated and undertaken in many women with *BRCA* mutations[21].

Online Methods

Patients and Samples

Cases—We used lymphocyte DNA from 1648 families with breast-ovarian cancer or breast cancer-only. These were ascertained from 24 genetics centres in the UK via the Genetics of Familial Breast Cancer Study (FBCS), which recruits women ≥18 years who have had breast cancer and/or ovarian cancer and have a family history of breast cancer and/or ovarian cancer. At least 97% of families are of European ancestry. Index cases from each family were screened and negative for germline mutations, including large rearrangements, in *BRCA1* and *BRCA2*. Informed consent was obtained from all participants and the research was approved by the London Multicentre Research Ethics Committee (MREC/01/2/18).

Breast-Ovarian Cancer Pedigrees: We included 911 unrelated index cases from breast-ovarian cancer pedigrees. The index cases were diagnosed with breast and/or ovarian cancer.

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Each family contained an individual with both breast and ovarian cancer or contained at least one case of breast cancer and at least one case of ovarian cancer with ≤1 intervening unaffected female relatives. Cases of ovarian cancer below the age of 20 were excluded from the analysis, as an appreciable proportion are likely to represent non-epithelial ovarian tumours, for example germ cell cancers. 271/911 probands had ovarian cancer (+/− breast cancer) and 617 probands had breast cancer only. The number of family members (including the probands) diagnosed with breast cancer and/or ovarian cancer, in the 911 breast-ovarian cancer pedigrees included in the analysis is illustrated in Supplementary Table 2.

Breast Cancer-only Pedigrees: We included 737 unrelated index cases from breast cancer-only pedigrees. The index case from each family was diagnosed with breast cancer, and had bilateral disease and/or a family history of breast cancer. There was no known case of ovarian cancer in any pedigree. The number of family members (including the probands) diagnosed with breast cancer, in the 737 breast cancer-only pedigrees included in the analysis is illustrated in Supplementary Table 2. The six cases of isolated breast cancer all had bilateral disease.

Samples and pathology information from mutation-positive Families: For families in which a mutation in *RAD51D* was detected, we sought DNA samples from relatives and all obtainable samples were genotyped for the family mutation. We also requested tumor material, pathology information, and receptor status in probands and affected relatives from the hospitals where they had been treated.

Controls—We used lymphocyte DNA from 1060 population-based controls obtained from the 1958 Birth Cohort Collection, an ongoing follow-up of persons born in Great Britain in one week in 1958. Biomedical assessment was undertaken during 2002-2004 at which blood samples and informed consent were obtained for creation of a genetic resource but phenotype data for these individuals is not available (http://www.cls.ioe.ac.uk/studies.asp?section=000100020003). At least 97% of the controls were of European ancestry.

Mutation analysis of *RAD51D*

We analysed genomic DNA extracted from lymphocytes for mutations by direct sequencing of the full coding sequence and intron/exon boundaries of *RAD51D*. Primer sequences and PCR conditions are given in Supplementary Table 1. The PCR reactions were performed in multiplex using the Qiagen Multiplex PCR Kit (Qiagen) according to the manufacturer's instructions. Amplicons were unidirectionally sequenced using the BigDyeTerminator Cycle sequencing kit and an ABI3730 automated sequencer (ABI Perkin Elmer). Sequencing traces were analysed using Mutation Surveyor software (www.softgenetics.com) and by visual inspection. All mutations were confirmed by bidirectional sequencing from a fresh aliquot of the stock DNA. Samples from members of *RAD51D* mutation-positive families were tested for the family mutation by direct sequencing of the appropriate exon.

In silico analyses of identified variants

We computed the predicted effects of *RAD51D* missense variants on protein function using PolyPhen[24] and SIFT[25]. All variants (intronic and coding) were analysed for their potential

effect on splicing. In the first instance, variants were analysed using two splice prediction algorithms NNsplice[26] and MaxEntScan[27], via the Alamut software interface (Interactive Biosoftware). If both NNsplice and MaxEntScan scores were altered by >20% (i.e. a wildtype splice-site score decreases and/or a cryptic splice-site score increases) three further prediction algorithms were utilised; NetGene2[28], HumanSplicingFinder[29], and Genscan[30]. A consensus decrease in a wildtype splice-site score and/or a consensus increase in a cryptic splicer-site score across all algorithms was considered indicative of disruption of normal splicing.

Tumor analysis

Representative tumor sections were stained with nuclear fast red and microdissected using a sterile needle and a stereomicroscope (Olympus SZ61, Tokyo, Japan) to ensure the proportion of tumour cells was >90%, as previously described[31]. DNA was extracted using the DNeasy kit (Qiagen) according to the manufacturer's instructions. DNA concentration was measured using the PicoGreen assay (Invitrogen), according to the manufacturer's instructions. *RAD51D* specific fragments encompassing the relevant mutations were PCR-amplified using the primers in Supplementary Table 1, and bidirectionally sequenced using the BigDyeTerminator Cycle sequencing kit and an ABI3730 automated sequencer (ABI Perkin Elmer). Sequence traces from tumor DNA were compared to sequence traces from lymphocyte DNA from the same individual.

Drug sensitivity

We used non-silencing *BRCA2* and *RAD51D* siGENOME siRNAs (Dharmacon, Lafayette, Colorado, USA). CAL51 and MCF7 cells were grown in DMEM (Gibco,, Invitrogen) supplemented with 10% (v/v) FCS (Gibco, Invitrogen). CHO RAD51D WT (51D1.3 clone) and CHO RAD51 dysfunctional (51D1 clone) cells were grown in αMEM (Gibco,, Invitrogen) supplemented with 10% FCS (Gibco,, Invitrogen). Cells were siRNA transfected using RNAiMAX (Invitrogen), plated in 96 well microtitre plates and then exposed to a titration of olaparib for 7 days. Media and drug was replenished every 3 days. After 7 days continuous culture, cell viability was estimated using Cell TitreGlo reagent (Promega Madison, Wisconsin, USA) and surviving fractions calculated as previously described[32].

Statistical methods

Statistical analyses were performed using STATA v11 software (StataCorp, College Station, Texas, USA). The frequency of mutations in cases and controls was compared using a two-sided Fisher's exact test. We estimated the *RAD51D* combined mutation frequency, the breast cancer risk ratio and the ovarian cancer risk ratio relative to non-*RAD51D* mutation carriers simultaneously using modified segregation analysis implemented in the pedigree analysis software MENDEL[33]. The analysis was based on breast and ovarian cancer occurrence in the combined dataset of families and controls. All individuals were censored at age 80 years, the age of their first cancer or their age of death or last observation, whichever occurred first. Females who had had bilateral prophylactic mastectomy were censored for breast cancer, and those who had had bilateral prophylactic oophorectomy were censored for ovarian cancer. Thus, only information on the first cancer was included in the primary analysis. We assumed that the breast incidence depends on the underlying genotype

through a model of the form: $\lambda(t) = \lambda_0(t)\exp(\beta x)$ where $\lambda_0(t)$ is the baseline incidence at age t in non-mutation carriers, β is the log risk ratio associated with the mutation and x takes value 0 for non-mutation carriers and 1 for mutation carriers. A similar model was assumed for the ovarian cancer incidences. Breast and ovarian cancers were assumed to occur independently, conditional on the genotype[22]. The overall breast and ovarian cancer incidences were constrained to agree with the population incidences for England and Wales in the period of 1993-1997[23], as described previously[34,35]. The models were parameterised in terms of the mutation frequencies and log-risk ratios for breast and ovarian cancer. Parameters were estimated using maximum likelihood estimation. Since *RAD51D* mutation screening was carried out in all index cases and controls we were able to incorporate information from all controls and the full pedigrees from all cases (including those without a *RAD51D* mutation) together with the segregation information from the families in which a *RAD51D* mutation was detected and genotyping was possible in relatives of the index case. To adjust for ascertainment, we modelled the conditional likelihood of all family phenotypes and mutation status of the index family member and other tested family members, given the disease phenotypes of all family members. For the controls we modelled the likelihood of the mutation status given they were unaffected. The variances of the parameters were obtained by inverting the observed information matrix. Log risk ratios were assumed to be normally distributed. Because this model does not explicitly incorporate the effects of other susceptibility genes, it assumes implicitly that the effects of *RAD51D* and other potential susceptibility genes can be regarded as independent, as in a multiplicative model. Power calculations were based on two-sided association testing with a significance level of α=0.05. We assumed that the observed frequency of truncating mutations in cases from breast-ovarian cancer families (0.88%) and controls (0.094%) reflects the true underlying mutation frequencies in the population, and that the effect calculated from the segregation analysis (OR=6.30) represents the true risk of ovarian cancer in the population. We assumed that the same ratio of truncating mutations: missense variants (predicted deleterious) would be detected in isolated cases of ovarian cancer as cases from breast-ovarian cancer families. We assumed that in association testing of mutation frequencies across 25,000 genes that the X^2 statistics will be normally distributed and we applied a Bonferroni correction for multiple testing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank all the patients and families that participated in the research. We thank Anita Hall, Darshna Dudakia, Jessie Bull, Rachel Linger and Anna Zachariou for their assistance in recruitment, Bernadette Ebbs for assistance in DNA extraction and running the ABI sequencers, Larry Thompson for the provision of cell lines and Ann Strydom for assistance in preparing the manuscript. We also thank. We are very grateful to all the clinicians and counsellors in the Breast Cancer Susceptibility Collaboration UK (BCSC) that have contributed to the recruitment and collection of the FBCS samples. The full list of BCSC contributors is in the Supplementary Note. This work was funded by Cancer Research UK (C8620/A8372 and C8620/A8857); US Military Acquisition (ACQ) Activity, Era of Hope Award (W81XWH-05-1-0204), Breakthrough Breast Cancer and the Institute of Cancer Research (UK). We acknowledge NHS funding to the NIHR Biomedical Research Centre. C.T. is an MRC-funded Clinical Research Fellow. A.C.A. is a Cancer Research UK Senior Cancer Research Fellow (C12292/A11174). We

acknowledge use of DNA from the British 1958 Birth Cohort collection, funded by the Medical Research Council grant G0000934 and the Wellcome Trust grant 068545/Z/02.

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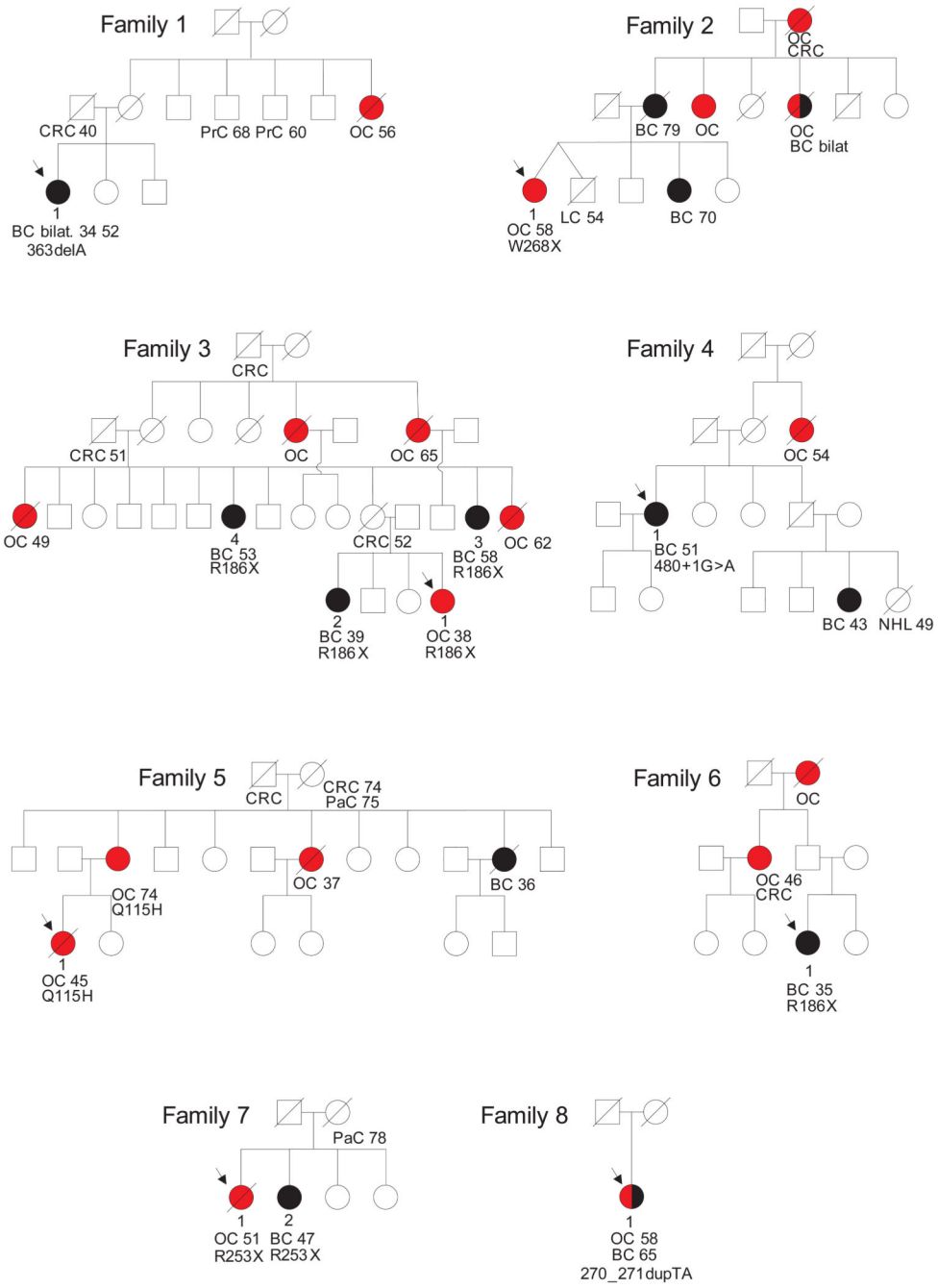

Figure 1.

Abridged pedigrees of eight families with *RAD51D* mutations. Individuals with ovarian cancer are shown as red circles, individuals with breast cancer are shown as black circles, other cancers are shown as unfilled circles or squares. Where known, the age of cancer diagnosis is under the individual, with two ages given for metachronas bilateral breast cancers. The relevant *RAD51D* mutation is given under the affected individuals analysed but not the unaffected individuals, to preserve confidentiality. BC, breast cancer; BC bilat.,

bilateral breast cancer; OC, ovarian cancer; CRC, colorectal cancer; LC, lung cancer; NHL, non-Hodgkin lymphoma; PaC, pancreatic cancer; Pr, prostate cancer.

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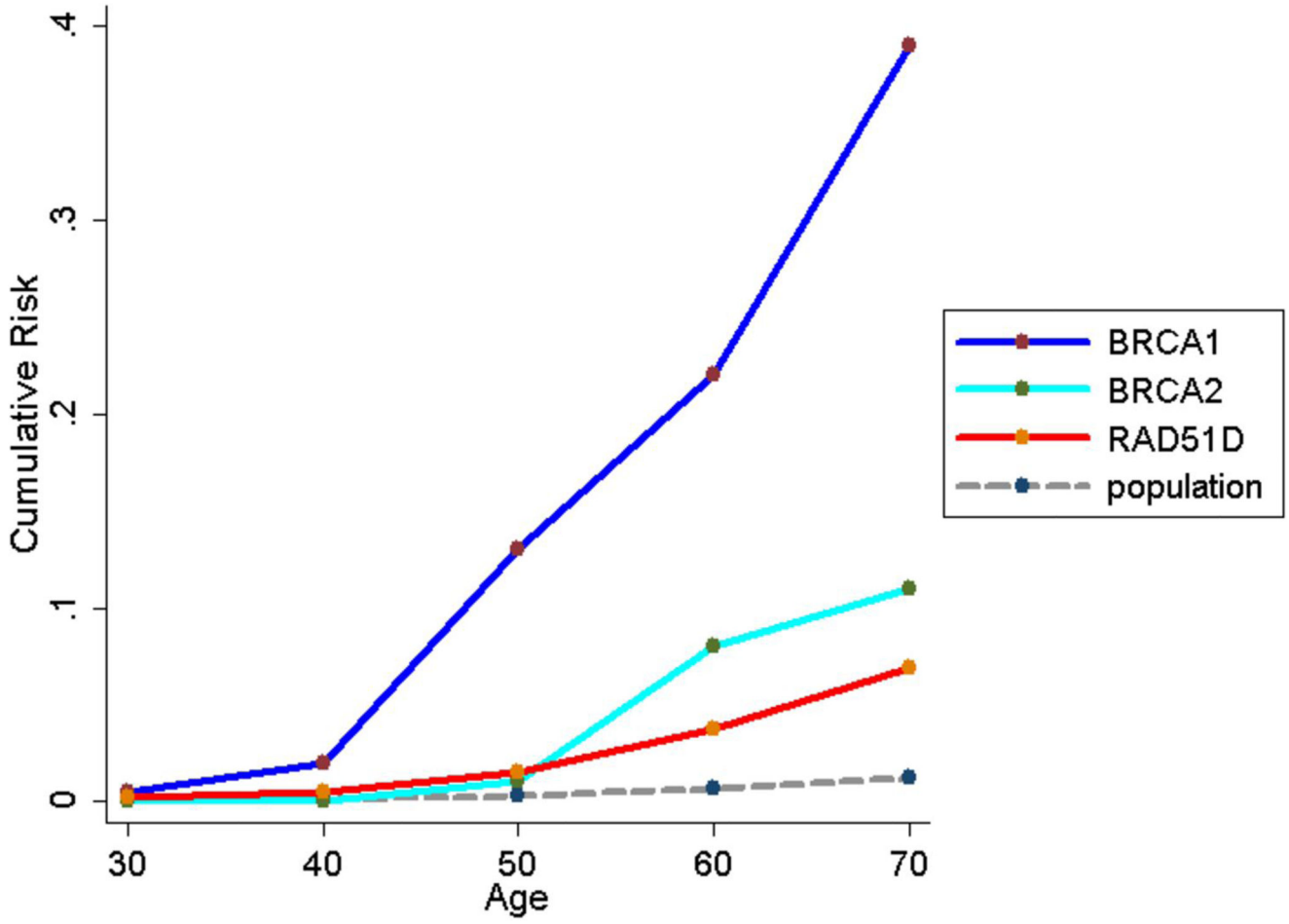

Figure 2.

Average age-related cumulative risk of ovarian cancer in *RAD51D* mutation carriers, *BRCA1* and *BRCA2* mutation carriers[22] and the population[23] .

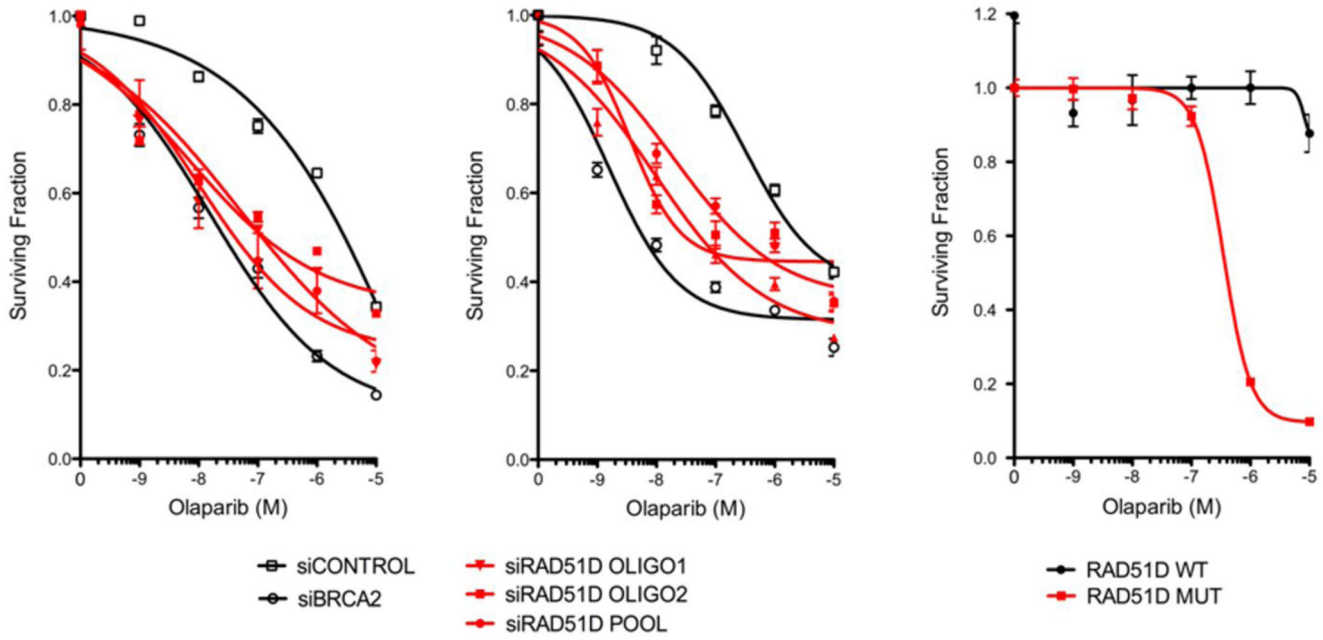

Figure 3.

Effect of *RAD51D* silencing on Olaparib sensitivity. CAL51 (a) or MCF7 (b) cells were transfected with siCONTROL, siRNA directed against *RAD51D* or siRNA directed against *BRCA2* and then treated with olaparib for 7 days before assaying for cell viability. Wild-type CHO cells or CHO cells mutated in *RAD51D* were treated with olaparib for 7 days before assaying for cell viability (c).

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Table 1

Cancer history and pathology in *RAD51D* mutation carriers

Family ID	Mutation*	Person ID	Cancer history (age at which cancer occurred, in years)	Pathology	Tumor analysis
FAM1	c.363delA	1	Breast cancer, left (34)	Invasive ductal carcinoma of no special type, grade 3	NA
			Breast cancer, right (52)	Invasive ductal carcinoma of no special type, grade 3	Loss of wildtype allele
FAM2	c.803G>A;W268X	1	Ovarian cancer (58)	Bilateral serous adenocarcinoma	Loss of wildtype allele
FAM3	c.556C>T;R186X	1	Ovarian cancer (38)	NA	NA
		2	Breast cancer (39)	High grade ductal comedo carcinoma in situ	NA
		3	Breast cancer (58)	Invasive carcinoma with medullary features	NA
		4	Breast cancer (53)	Invasive ductal carcinoma of no special type	NA
FAM4	c.480+1G>A	1	Breast cancer (51)	Invasive ductal carcinoma of no special type, grade 3	NA
FAM5	c.345G>C;Q115H**	1	Ovarian cancer (45)	Bilateral serous adenocarcinoma	NA
		2	Ovarian cancer (74)	NA	NA
FAM6	c.556C>T;R186X	1	Breast cancer (35)	Invasive ductal carcinoma of no special type, grade 3	NA
FAM7	c.757C>T;R253X	1	Ovarian cancer (51)	Differentiated endometrioid adenocarcinoma	NA
		2	Breast cancer (47)	NA	NA
FAM8	c.270_271dupTA	1	Ovarian cancer (58)	Differentiated adenocarcinoma	Loss of mutant allele
			Breast cancer (65)	Invasive ductal carcinoma of no special type, grade 3	Reduction of wildtype allele
Control	c.748delC		NA	NA	NA

*Mutation nomenclature corresponds to Ensembl Transcript ID ENST00000345365

**This mutation is at the final base of exon 4, disrupts the splice-site and results in skipping of exons 3 and 4. Person IDs correspond to Fig. 1. NA: not available.