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ORIGINAL ARTICLE

FANCM, RAD1, CHEK1 and *TP53I3* act as *BRCA*-like tumor suppressors and are mutated in hereditary ovarian cancer

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Abstract

Although 25% of ovarian cancer cases are due to inherited factors, most of the genetic risk remains unexplained. We previously identified candidate genes through germline whole exome sequencing of *BRCA1/BRCA2* negative ovarian cancer patients with familial risk. Here, we performed functional assessment to determine whether they act as *BRCA*-like tumor suppressors.

Seven candidate risk genes were targeted by siRNA for mRNA depletion followed by functional assays for clonogenic survival, cytotoxicity to DNA damaging agents, and involvement in homologous recombination repair. *BRCA1* and *BRCA1* were targeted as standards for loss of function outcome.

Knockdown of various candidate genes led to tumor suppressor phenotypes also observed in *BRCA1/BRCA2* deficient cells. Deficiency of *CHEK1, FANCM* and *TP53I3* led to reduced homologous recombination repair efficiency. Knockdown of *RAD1, CHEK1* or *FANCM* led to a decrease in cellular viability and cells deficient in *CHEK1, RAD1* or *TP53I3* displayed increased sensitivity to cisplatin.

Functional studies of candidate genes identified by whole exome sequencing complements bioinformatics techniques and aid the implication of novel risk loci. The results of this study suggest that genes found mutated in hereditary ovarian cancer, *FANCM, RAD1, CHEK1* and *TP53I3*, act as *BRCA*-like tumor suppressors.

Keywords Hereditary breast and ovarian cancer, Genetic risk, Cancer predisposition.

Introduction

Despite the large heritable component to OVCA, the majority of underlying genetic risk remains unexplained [1]. OVCA is rare, displays variable penetrance, and has a high degree of underlying genetic heterogeneity. The clinical implications of a novel risk locus, is therefore difficult to accurately establish through case control associative studies [2,3]. Additionally, implicating a variant based on segregation is not ideal due to incomplete penetrance and lack of informative family members. Whole exome/genome sequencing (WES/WGS) of affected individuals with compelling family histories is a promising approach for the identification of putative germline novel risk loci. However, bioinformatic tools alone are not sufficiently accurate to direct clinical decisions regarding novel genetic loci. Instead, these putative risk loci require the support of functional assessment.

We previously carried out WES on OVCA patients considered at high risk of genetic inheritance, but with no known pathogenic variant in *BRCA1* or *BRCA2* [4], the two most commonly mutated genes in HBOC (Hereditary Breast and Ovarian Cancer). We identified 11 loss of function (LOF) (stop

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gain, frameshift) variants in genes not yet implicated in hereditary OVCA risk, but whose protein products are involved in DNA repair and/or cell cycle control, the most commonly mutated pathways in HBOC. The current study aimed to functionally assess seven of these candidate genes to identify those with *BRCA*-like tumor suppressor qualities. Specifically, we assessed *FANCM, CHEK1, MCM4, RAD1,* and *REC8* because of their conservation and cancer associated phenotypes in mouse models, which include embryonic lethality in homozygote knockouts and increased cancer incidence in heterozygotes [5–9]. We assessed *TP53I3* because our cohort included a rare LOF mutation in this gene in two unrelated individuals. We also assessed *HMMR* as it forms a complex with *BRCA1*/*BRCA2*, and common missense variations in this gene modifies the penetrance of breast cancer risk in *BRCA1* pathogenic mutation carriers [10]. Positive controls for functional analyses included established HBOC risk genes *BRCA1* and *BRCA2* (high risk) and *ATM, CHEK2, RAD51D* (moderate risk), which are featured on current HBOC testing panels.

Materials and methods

Patient accrual, whole exome sequencing and SNP assessment

We performed whole exome sequencing on a sample of 48 women diagnosed with ovarian cancer, suspected to be at high risk of hereditary predisposition, but with no known pathogenic mutation in *BRCA1* or *BRCA2*. Materials and detailed methods regarding patient accrual, whole exome sequencing, in silico SNP assessment and candidate gene analysis were previously reported and can be accessed here: (http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0178450, PMID: 28591191) [4]. VCF files available at Figshare; https://figshare.com/s/7471a180cd770aeda2fd.

Cell line and culture

We used HeLa cells for all functional experimentation. HeLa were chosen based on various necessary criteria including having an intact homologous repair pathway [11,12], a doubling capacity of every 24 h, and epithelial in origin. We originally tested five various ovarian cell lines, however three had no P53 wild type activity necessary for the employed assays. The remaining two were found to be cervical in origin after cell line authentication (Applied Genomics Technology Center at Wayne State University). HeLa cells stably transfected with the DR-GFP homologous recombination analysis plasmid (M. Jasin, Memorial Sloan Kettering Cancer Institute, New York, NY) were a kind gift from Dr. Jeffery Parvin of Ohio State University. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (HyClone, GE Cat: SH30396.03), NaHCO3 (3.7 g/L) 1% penicillin strep (Gibco Life Technologies Cat: 15140-122) and incubated at 37 °C 5% CO_2. For passaging, cells were harvested using trypsin, and split 1:6. Freeze downs of aliquots were maintained in a freezing media of 10% DMSO, 50% DMEM, 40% FBS and stored at −140 °C.

Protein detection and quantification

Western blot was employed to assess protein expression and quantification to ensure sufficient knockdown by siRNA (Fig. A.1). Cells were lysed, and proteins solubilized using ice cold Radio Immunoprecipitation Assay Buffer (RIPA) supplemented with protease and phosphatase inhibitors. Proteins were reduced and denatured by adding 4× Laemmli buffer plus β-mercaptoethanol to lysates (9:1 ratio, respectively) in addition to boiling at 95 °C for 5 min using thermocycler. Fifty to seventy micrograms of denatured whole cell lysate were loaded into each well of a polyacrylamide gel and separated by electrophoresis, followed by a wet transfer to a nitrocellulose membrane using electrical current. Membranes were blocked with 5% bovine serum albumin (BSA) in Tris Buffered Saline with Tween 20 (TBST) before being incubated with protein specific antibodies. Images were obtained by LI-COR Odyssey Blot Imager and protein expression was quantified using ImageJ software and normalized to appropriate loading control (ACTIN for smaller proteins, VINCULIN for high molecular weight proteins). A complete list of antibodies is provided in the appendix (Table A.1).

Homologous recombination repair assay

The homologous recombination repair assay is a sensitive method developed to measure Homologous Recombination Repair (HRR) pathway efficiency following double strand breaks (DSB) [13]. HeLa cells were stably transfected with the pDR-GFP plasmid that contains two inactive GFP alleles. One GFP allele is inactive due to the presence of an additional sequence that contains the 18 bp restriction enzyme recognition site for I-SceI, and the other GFP allele is inactive due to a truncating mutation [13]. When a second plasmid encoding the enzyme I-SceI is transiently transfected into cells containing this plasmid, the I-SceI restriction site is cleaved, creating a DSB. The break is repaired by the cells' endogenous HRR pathway using the second inactive GFP allele as a template. Therefore, the amount of GFP following I-SceI DSB induction provides a proxy for the activity of the HRR pathway.

The following protocol is an adaptation of a previously described protocol [11]. HeLa DR-GFP were harvested with trypsin, counted using a hemocytometer and reseeded at 40,000 into each well of a 24 well plate. Following 24 h, media was replaced with 450 µl serum and antibiotic free media and cells were transiently transfected with I-SceI Transfection expression vector pCBASce [13]. 0.5 µg of plasmid and 15 pmol siRNA were co-transfected using Lipofectamine 3000 (Thermo Fisher Cat: L3000015) according to manufacturer's protocol. As a negative control, cells were transfected with pcDNA3 empty vector (Invitrogen) in place of I-SceI to measure background GFP signal. As a positive control, cells were transfected with scramble siRNA plus I-SceI. Each condition was performed in triplicate. Forty-eight hours post transient transfection of the I-SceI containing plasmid, cells were visualized for GFP signals using fluorescence microscopy (Olympus 1 × 71) followed by harvesting and quantitative analysis by flow cytometry (BD FACSCanto II and BD FACS Diva Software v8.0.1). Gating procedure was set to select singlets, live (DAPI) and GFP (FITC) positive cells. A *t*-test was employed to compare the amount of GFP in cells transfected with a siRNA

knockdown of a gene of interest to its respective siRNA scramble positive control, controlling for background GFP (negative control). A complete list of siRNAs is provided in Supplementary data.

Clonogenic survival assay

Cells were trypsinized and 350,000 were plated in a 60-mm tissue culture dishes and incubated overnight at 5% CO_2 and 37 °C. Cells were then transfected with siRNA using Oligofectamine (Invitrogen Cat# 12252-011) according to the manufacturer's protocol. Twenty-four hours after transfection, cells were re-counted, and 100 or 300 cells were reseeded in triplicate of a 6 well plate and placed back in incubator. Forty-eight hours after siRNA knockdown, wells were replaced with serum free media containing a DNA damaging reagent such as cisplatin, etoposide, olaparib or mock control. Drug concentration and exposure were determined by optimizing for an $IC_{50,}$ which was determined by the amount and duration of treatment necessary to cause 50% of cells to die (cisplatin = 2 μM for 2 h, etoposide = 10 μM for 4 h, and olaparib 10 μM for 4 h, data not shown). Cells were rinsed twice with serum free media before adding back fresh media and placing back in incubator for 10 days until colonies had formed (>50 cells per colony). For fixation and staining, medium was removed, and cells washed with PBS before adding add 2 ml of 1:7 acetic acid/methanol fixation solution for 5 min followed by 2 ml 0.5% crystal violet solution for 2 h at room temperature. Colonies were counted both by eye and with a colony counter (GELCOUNT, Oxford Optronix). Plating efficiency (PE) was calculated as the number of colonies formed divided by the number of cells seeded × 100%. Survival after drug treatment was determined by calculating the number of colonies formed divided by the number of cells seeded × 100%, adjusted for PE.

Statistical analysis

Statistical analyses of homologous recombination repair and clonogenic assays were carried out using R statistical software (version 3.4.1). To reduce inflation of Type II error, we analyzed approximately six replicates per condition. Variability in assay performance including siRNA knockdown was controlled for by pooling replicates for each condition across all repeated assays (each assay was performed on two or three separate occasions with each condition in duplicate or triplicate). Prior to the analysis, we checked for and did not find outliers (i.e., data points greater than 3 standard deviations from the mean for each variable). We next investigated the distribution of replicates for each tested condition (reduction in HRR efficiency, plating efficiency, and adjusted survival rate after drug treatment following siRNA knockdown). First, we calculated Shapiro–Wilk's test, which tests the null hypothesis that a sample distribution was drawn from a normally distributed population. Next, we assessed skewness and kurtosis for each gene per condition. For small samples ($n < 50$), *z*-scores less than 1.96 for either skewness or kurtosis suggests a normal distribution [14]. The overall pattern of results generated from the HRR and clonogenic assays indicated normal distributions (results available upon request).

Table 1 HRR efficiency after siRNA knockdown *M* = Mean% of Green Fluorescent Protein (GFP) positive cells; *SD* = Standard deviation; *MD* = mean difference as compared to scramble control; *t* = statistical test for mean difference; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; *p*-values in bold are significant.

	Gene	*M*	*SD*	*MD*	*t*	*p*
	Scramble	97.4	2.58	—	—	—
Panel	ATM	51.9	13.43	−45.5	6.72	**0.006**
	BRCA1	27.5	8.32	−69.9	21.45	**0.000**
	BRCA2	8.6	5.02	−88.8	39.97	**0.000**
	CHEK2	70.3	9.96	−27.1	5.98	**0.003**
	RAD51D	62.8	12.61	−34.7	5.452	**0.011**
Non-panel	FANCM	43.3	6.68	−54.1	21.53	**0.000**
	CHEK1	28.2	14.04	−69.2	11.95	**0.000**
	RAD1	108.7	7.57	11.2	−2.52	0.119
	REC8	82.6	5.68	−14.8	6.40	**0.000**
	TP53I3	64.4	13.55	−33.0	7.55	**0.000**

Paired sample *t*-tests were conducted to identify mean differences in the survival rates for each siRNA knockdown as compared to the scramble siRNA control for each condition.

Results

Knockdown of various candidate genes led to reduced homologous recombination repair efficiency

Short interfering RNA (siRNA) depletion of panel genes tested (*BRCA1, BRCA2, ATM, CHEK2* and *RAD51D*) led to a significant decrease in HRR efficiency (Fig. 1). Knockdown of *BRCA1* and *BRCA2* led to the greatest reduction in HRR efficiency (approximately 69.9% and 88.8% less compared to scramble control respectively; see Table 1). *ATM, CHEK2* and *RAD51D* knockdown led to a more moderate reduction in HRR efficiency (45.5% 27.1%, and 34.7% reduction, respectively), consistent with their roles as moderately penetrant genes. Additionally, siRNA depletion of candidate genes *REC8, TP53I3, CHEK1* and *FANCM* lead to a reduction in HRR ($p < 0.001$). The greatest reduction among candidate genes was observed with *CHEK1* with a reduction of 69.2%. The next largest reduction in HRR efficiency was observed with *FANCM* (54.1%). Knockdown of *TP53I3* led to a reduction of 33%, and *REC8* showed a reduction of 14.8%. The effect of siRNA depletion of *RAD1* on HRR efficiency was not significant ($p = 0.119$) but trended towards an increase (11.2%).

Knockdown of *RAD1, CHEK1* or *FANCM* led to a decrease in cellular viability

One key genetic mechanism in the process of tumorigenesis is the loss of heterozygosity (LOH) at tumor suppressor loci leading to the "inactivation" of genes required for the regulation of cell growth and differentiation [15]. Functional loss of genes essential for genomic stability is known to encourage LOH [16] and is associated with increased cancer risk. The

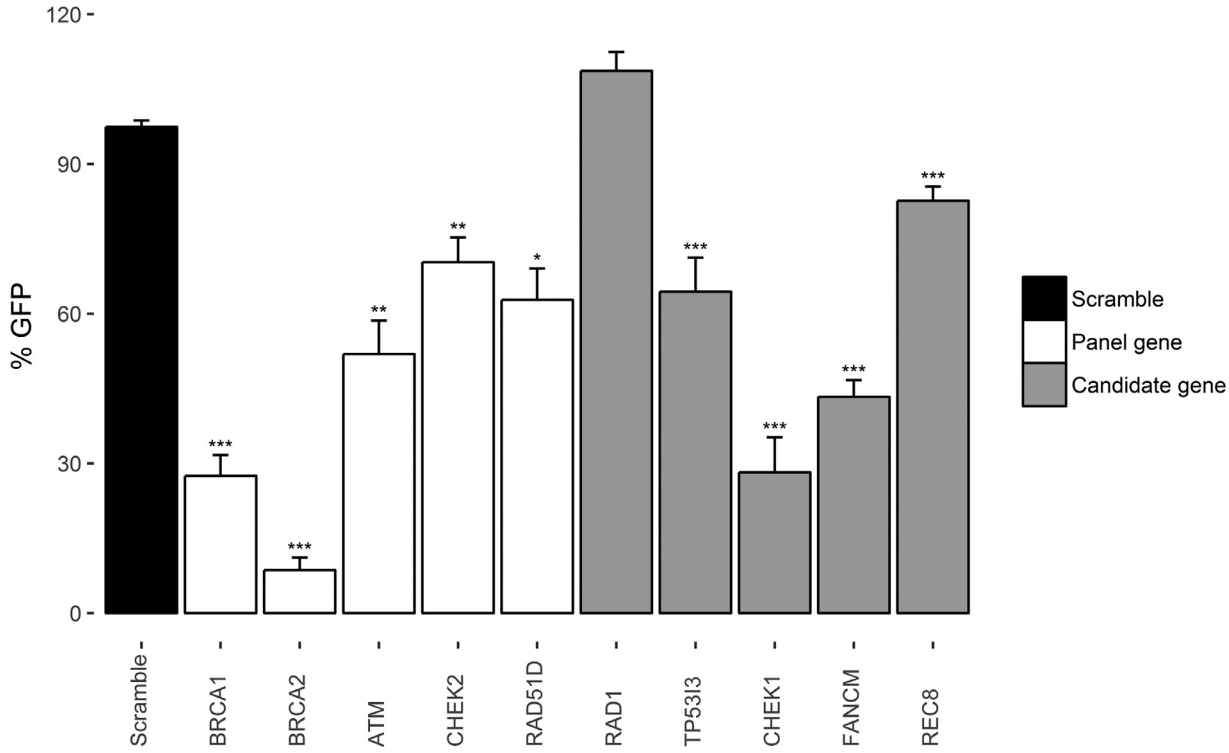

Fig. 1 HRR efficiency after siRNA knockdown. GFP (green fluorescent protein) signal proxies for DNA repair by homologous recombination. For comparison purposes, the data across various experiments were normalized to 1 (100% GFP) for the negative control (scramble siRNA). The loss in GFP signal after siRNA transfection indicates a loss of HRR pathway efficiency. *BRCA1* and *BRCA2* are typical controls for this assay due to their high impact on the HRR pathway. The inclusion of *ATM, CHECK2* and *RAD51D* validate the sensitivity of this assay to identify genes of moderate impact.

clonogenic survival assay tests the ability of a single cell to form a colony and is a well-established method to determine the importance of a gene to genomic stability and cellular survival. To identify which of the candidate genes are also involved, we compared the clonogenic survival of cells after siRNA knockdown of candidate and positive control panel genes to a scramble siRNA control (Fig. 2A, Table 2, Plating efficiency).

Among panel genes tested, knockdown of *BRCA1, BRCA2* or *ATM,* led to significant a loss of clonogenic survival (reported as plating efficiency after knockdown). There was no loss of cellular viability observed for panel genes *CHEK2* or *RAD51D*. For candidate gene *CHEK1*, the loss of clonogenic survival was 13.8%, similar to *BRCA1* and *BRCA2* (mean survival of 16.5%, and 8.8%, respectively). SiRNA knockdown of candidate gene *RAD1* led to a reduction in clonogenic survival that was similar to that observed with *ATM* (mean survival of 23.3% vs 29.6%, respectively). Finally, siRNA depletion of *FANCM* led to the greatest loss in clonogenic survival, with a mean plating efficiency of 2%.

Cells deficient in *CHEK1, RAD1, TP53I3* or *REC8* display increased cytotoxicity to DNA damage

Clonogenic survival assays are also a well-established tool for assessing cell sensitivity to DNA damaging treatments, such as ionizing radiation or chemotherapeutic drugs, providing insight for the assessment of a targeted therapy. Genomic stability is directly related to a cell's DNA repair efficiency, and cells deficient in DNA repair mechanisms display greater sensitivity to DNA damaging reagents. Chemotherapeutic drugs cisplatin, etoposide, and olaparib exploit this vulnerability, and cells deficient in *BRCA1* or *BRCA2* are especially sensitive to these reagents [17–19]. To assess whether, and to what extent, loss any of the candidate genes would also lead to increased sensitivity to these reagents, we compared the clonogenic survival of cells with targeted siRNA depletion of candidate and panel genes to a scramble siRNA, followed by exposure to cisplatin, etoposide, or olaparib. Cells were exposed to the drugs 48 h post siRNA knockdown. The clonogenic survival after drug exposure for each condition was adjusted to the plating efficiency observed with the same siRNA knockdown and without drug exposure. The assessment of candidate gene *FANCM* to these reagents was not possible due to the extreme loss of cellular viability that occurred after siRNA depletion. Results are depicted in Fig. 2B–D and Table 2.

Cisplatin generates interstrand cross links (ICLs; covalent bonds between both strands of a DNA duplex). ICLs inhibit crucial processes such as DNA replication, ultimately leading to chromosomal instability. ICLs are repaired by HRR, so that cells deficient in this pathway are highly sensitive to DNA-damaging agents such as cisplatin. As expected, all HBOC panel genes included in this assay *(BRCA1, BRCA2, ATM, CHEK2,* and *RAD51D*) were demonstrated to be involved in

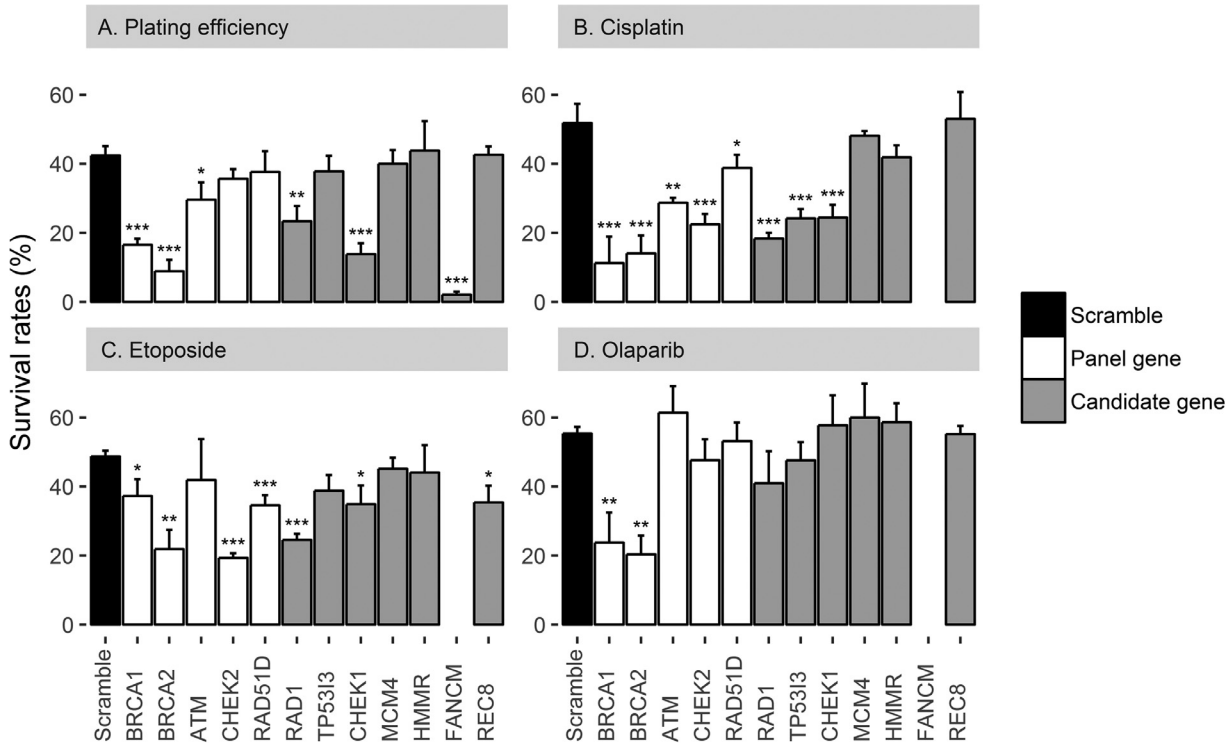

Fig. 2 Clonogenic survival rates after drug exposure by siRNA knockdown A. Plating efficiency (cell survival after re-plating) by siRNA knockdown with no drug exposure. B. Cell Survival after exposure to cisplatin by siRNA knockdown adjusted for cell loss after re-plating (plating efficiency). C. Cell Survival after exposure to etoposide by siRNA knockdown adjusted for cell loss after re-plating. D. Cell Survival after exposure to olaparib by siRNA knockdown adjusted for cell loss after re-plating. Paired sample t-tests were conducted to identify mean differences in the survival rates for each siRNA knockdown as compared to the scramble siRNA control for each condition. $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; *p*-values in bold are significant. Error bars represent a 0.5 standard deviation.

HRR, and ICL repair as targeted by siRNA against their respective mRNA products led to an increase in cytotoxicity with cisplatin exposure (Fig. 2B, Table 2). Cells with knockdown of candidate genes (*CHEK1, RAD1,* or *TP53I3*) also displayed significant sensitivity to cisplatin exposure equal or greater to that observed in the moderate impact panel genes (*ATM, RAD51D* and *CHEK2*; Fig. 2B, Table 2). Loss of *RAD1* led to the most sensitivity among candidate genes with a mean viability of 18.3%, representing 33% increased cytotoxicity as compared to the scramble control ($p < 0.001$). Depletion of *CHEK1* and *TP53I3* both led to a mean increase in cisplatin cytotoxicity of approximately 27% ($p < 0.001$). Cisplatin cytotoxicity did not increase after knockdown of *REC8, HMMR* and *MCM4*.

Etoposide is a topoisomerase II (topoII) alpha inhibitor approved for clinical use as a chemotherapeutic reagent. Topoisomerase II enzymes are responsible for simultaneously cleaving both strands of the DNA double helix for the management of entangled and supercoiled DNA. These enzymes are essential for DNA replication. Inhibition of topoII by etoposide prevents the re-ligation of cleaved DNA and therefore leads to DNA DSBs. Etoposide also results in single-strand DNA breaks (SSBs), because it prevents the re-ligation of the stands independently of each other [20]. Both *BRCA1* and *BRCA2* deficient cells have been described as sensitive to etoposide treatment [18]. Consistent with these reports, both *BRCA1* and *BRCA2* deficient cells displayed significant sensitivity to etoposide in this assay. Knockdown of *CHEK2* or *RAD51D* also led to increased etoposide sensitivity (with approximately 30% and 14% increased cytotoxicity, respectively). Among non-panel candidate genes, *CHEK1, RAD1* and *REC8* knockdown resulted in increased cytotoxicity in response to etoposide. *CHEK1* deficient cells displayed a mean survival of 34.9% ($p = 0.024$), *RAD1* 24.5% ($p < 0.001$) and *REC8* 35.4% ($p = 0.018$), which is an increase of approximately 14%, 24% and 13% in cytotoxicity versus the scramble control respectively (Fig. 2C, Table 2).

Olaparib is a PolyADP-ribose polymerase (PARP) inhibitor approved for clinical as an adjunct to platinum-based therapies in patients with *BRCA1* or *BRCA2* deficiencies. Tumor cells with mutant *BRCA1* and/or *BRCA2* have been demonstrated to be up to 1000 times more sensitive to PARP inhibitors as compared to WT cells [19,21]. Cancer cells deficient in HRR are more dependent on the PARP proteins, which are involved in Base Excision Repair (BER). Inhibiting this pathway leads to synthetic lethality as the cells lose its back up mechanism to repair DNA. In clinical trials, the use of PARP inhibitors have shown to improve progression-free survival when added to the treatment of women with breast or ovarian cancer responsive to platinum, which induces DSBs [22–24]. Olaparib used as a monotherapy has shown to be effective in patients with germline *BRCA1/2* mutation and advanced cancer [25,26]. In this experiment, cells were treated with olaparib in the absence of cisplatin, and increased

Table 2 Statistical data for clonogenic survival rates after knockdown alone (plating efficiency) or in the presence of chemotherapeutic reagent; cisplatin, etoposide or olaparib. *M* = mean% survival; *SD* = Standard deviation; *MD* = mean difference as compared to scramble control; *t* = statistical test for mean difference; p-values in bold are significant.

		Condition (survival rates)																	
		Plating efficiency				Cisplatin				Etoposide				Olaparib					
	Gene	*M*	*SD*	*MD*	*t*	*p*	*M*	*SD*	*MD*	*t*	*p*	*M*	*SD*	*MD*	*t*	*p*	*M*	*SD*	*MD*	*t*	*p*
	Scramble	42.4	5.44	—	—	—	51.8	11.19	—	—	—	48.8	3.30	—	—	—	55.3	3.94	—	—	—
Panel	ATM	29.6	10.06	−12.8	−2.55	**0.044**	28.7	2.93	−23.1	−4.74	**0.003**	41.9	23.77	−6.9	−0.70	0.511	61.4	15.42	6.0	0.77	0.495
	BRCA1	16.5	3.54	−25.9	−9.78	**0.000**	11.2	15.38	−40.6	−5.23	**0.001**	37.3	9.71	−11.5	−2.74	**0.033**	23.7	17.54	−31.6	−4.31	**0.006**
	BRCA2	8.8	6.59	−33.6	−9.62	**0.000**	14.0	10.44	−37.8	−6.05	**0.000**	21.9	11.13	−26.9	−5.21	**0.004**	20.4	10.85	−35.0	−6.84	**0.001**
	CHEK2	35.7	5.54	−6.7	−2.12	0.060	22.4	6.12	−29.4	−5.52	**0.001**	19.3	2.66	−29.4	−16.37	**0.000**	47.7	12.10	−7.7	−1.36	0.235
	RAD51D	37.7	11.94	−4.7	−0.88	0.406	38.8	7.61	−13.0	−2.36	**0.043**	34.6	5.77	−14.2	−5.22	**0.001**	53.2	10.68	−2.2	−0.47	0.656
Non-panel	FANCM	2.1	1.83	−40.3	−17.2	**0.000**	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	CHEK1	13.8	6.37	−28.6	−8.37	**0.000**	24.4	7.38	−27.4	−5.00	**0.001**	34.9	10.83	−13.8	−3.00	**0.024**	57.7	17.46	2.4	0.33	0.754
	HMMR	43.8	17.09	1.4	0.19	0.855	41.9	6.99	−10.0	−1.85	0.100	44.1	15.84	−4.7	−0.71	0.508	58.7	10.88	3.3	0.70	0.507
	MCM4	40.0	7.87	−2.4	−0.61	0.554	48.1	2.71	−3.7	−0.78	0.465	45.1	6.40	−3.6	−1.23	0.256	60.0	19.67	4.7	0.57	0.592
	RAD1	23.3	8.82	−19.1	−4.51	**0.002**	18.3	3.31	−33.5	−7.03	**0.000**	24.5	3.55	−24.2	−12.25	**0.000**	41.0	18.53	−14.4	−1.86	0.117
	REC8	42.6	4.78	0.2	0.06	0.952	53.0	15.54	1.2	0.14	0.891	35.4	9.64	−13.3	−3.21	**0.018**	55.2	4.70	−0.2	−0.05	0.963
	TP53I3	37.8	8.98	−4.6	−1.07	0.315	24.2	5.44	−27.6	−5.44	**0.001**	38.8	9.11	−10.0	−2.32	0.069	47.6	10.51	−7.7	−1.69	0.139

cytotoxicity was specific to *BRCA1* and *BRCA2* deficient cells (Fig. 2D, Table 2).

Discussion

Candidate loci identified by WES and bioinformatics techniques were followed up by functional assessment using sensitive cell-based laboratory techniques. This approach identified four novel genes, *FANCM, CHEK1, RAD1* and *TP53I3,* as having the *BRCA*-like phenotype typically observed in tumor suppressor genes commonly mutated in the germline of women with inherited risk of breast and/or ovarian cancer. SiRNA knockdown of *FANCM* led to a reduction in homologous recombination repair and large loss of clonogenic survival similar to that observed in *BRCA1* or *BRCA2* deficient cells. Because of the large loss in cell survival after knockdown, *FANCM* could not be assessed for cytotoxicity to cisplatin, olaparib, or etoposide after knockdown. However, the functional data from this study, along with the family pedigree of the carrier with a c.5791C>*T* nonsense FANCM variant (Fig. B.1), association with familial breast cancer [27] and high grade serous ovarian cancer [28] in recent literature is supportive of a high risk allele. Additionally, *FANCM* null mice have a decreased life span and increased cancer incidence [5].

Candidate gene *CHEK1,* like panel gene *CHEK2,* encodes for a serine/threonine protein kinase required for checkpoint-mediated cell cycle arrest and activation of DNA repair by homologous recombination repair. This gene is highly conserved, and the c.1564-1565insA frameshift variant identified by WES in this study sample is novel. In this study, siRNA knockdown of *CHEK1* led to a decrease in HRR efficiency, reduction in cellular viability, and increased sensitivity to cisplatin and etoposide, similar to that of observed in *BRCA1/BRCA2* deficient cells. These results are consistent with mouse model phenotypes describing homozygote nulls as embryonic lethal, and heterozygote knockouts displaying enhanced tumorigenesis [29]. Recently, *CHEK1* has been identified as an important biomarker for chemotherapy response in breast cancer [30] and the deletion of CHEK1 is a common genetic event that occurs in the beginning stages of breast cancer development [31]. Loss of heterozygosity is likely to have occurred in the carrier of the *CHEK1* frameshift. Unfortunately, the patient declined tumor tissue usage, and this could not be confirmed. The fact that *CHEK1* has not already been implicated in hereditary risk of ovarian or breast cancer is likely because pathogenic variants in this gene are extremely rare.

Another novel loss of function variant was uncovered in *RAD1. RAD1* null mice display embryonic lethality in homozygotes and larger, more numerous, earlier onset skin tumors with DMBA-TPA treatment in heterozygotes [8]. In this study, siRNA knockdown of *RAD1* led to decreased cellular viability and increased sensitivity to cisplatin and etoposide, similar to that observed with knockdown of *BRCA1* and *BRCA2.* However, *RAD1* depletion did not lead to a reduction in HRR efficiency. One possible explanation is that RAD1 plays a role in micro homology-mediated end joining (MMEJ) for the repair of ionizing radiation and chemicals that induce DSBs [32], such that cells depleted of *RAD1* may be more reliant on the HRR pathway for DSB repair. The observation of a germline

RAD1 LOF variant in an OVCA patient considered at high risk of genetic inheritance, plus the results of this study's functional analyses make a compelling case for this gene as a risk factor. The carrier of the *RAD1* frameshift variant developed OVCA at the age of 65, and had a family history of colon, breast ($n=2$), prostate, lung, and leukemia, all of which on one parental side of the family (Fig. C.1), which is indicative of a highly penetrant germline risk variant. Unfortunately, segregation analysis was not possible since DNA samples from her deceased family members are not available.

TP53I3 is an oxidoreductase-like protein and an inducer of ROS, that is transcriptionally activated by the tumor suppressor *TP53* and likely to be involved in *TP53*-mediated apoptosis [33]. A nonsense SNP in this gene was observed twice in this study sample among unrelated individuals, despite its rarity (rs145078765, p.S252*, MAF= 0.0009) The functional analysis of this gene during this study indicated that loss of *TP53I3* leads to a moderate reduction in HRR efficiency as well as increased cell sensitivity to cisplatin. Another candidate gene assessed in this study was *REC8*, which encodes for a cohesin complex protein required for the structural maintenance of chromosomes during meiosis. Cohesions are necessary to join sister chromatids together until DNA replication is complete. SiRNA reduction of *REC8* led to a moderate decrease in DNA repair by homologous recombination and increased sensitivity to etoposide. Recently, investigators identified *REC8* as a tumor suppressor gene epigenetically downregulated in gastric cancer [34]. Another study demonstrated that epigenetic silencing of *REC8* was robustly associated with PI3K pathway alterations in thyroid tumors, possibly enhancing the oncogenic properties of the PI3K/AKT/mTOR pathway, which is important to cell cycle regulation [35].

In conclusion, we previously discovered multiple loss of function variants in candidate genes through the whole exome sequencing of women with high familial risk but no known pathogenic mutation in any of the currently established risk genes. The functional analyses employed in this study suggest that four of these genes, *FANCM, RAD1, CHEK1* and *TP53I3,* act as tumor suppressors in a *BRCA*-like manner. Most of the genetic risk underlying hereditary breast and ovarian cancer remains unexplained since mutations that incur clinically relevant risk are rare, making implication by association unlikely. Therefore, whole exome sequencing of a high risk cohort followed by functional analyses are invaluable to closing the gap in the missing heritability of this disease.

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Conflict of interest statement

All authors of this manuscript immediately certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cancergen.2019.04.061.

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