Frequency of mutations in mismatch repair genes in a population-based study of women with ovarian cancer

T Pal*,[1],[12], MR Akbari[2],[12], P Sun[2], J-H Lee[1], J Fulp[1], Z Thompson[1], D Coppola[1], S Nicosia[3], TA Sellers[1], J McLaughlin[4],[5], HA Risch[6],[7], B Rosen[8],[9], P Shaw[10], J Schildkraut[11] and SA Narod[2]

[1]Departments of Cancer Epidemiology, Biostatistics, Anatomic Pathology and Experimental Therapeutics, Moffitt Cancer Center, MRC-CANCONT, 12902 Magnolia Drive, Tampa, FL 33612, USA; [2]Womens College Research Institute, Womens College Hospital, University of Toronto Hospital, 790 Bay Street, 7th Floor, Toronto, ON, Canada M5G 1N8; [3]Department of Pathology and Cell Biology, University of South Florida College of Medicine, 12901 Bruce B. Downs Boulevard, Tampa, FL 33612, USA; [4]Samuel Lunenfeld Research Institute, School of Public Health, University of Toronto, 600 University Avenue, Toronto, ON, Canada M5G 1X5; [5]Department of Public Health, Dalla Lana School of Public Health, University of Toronto, 600 University Avenue, Toronto, ON, Canada M5G 1X5; [6]Department of Epidemiology and Public Health, School of Public Health, Yale University, 60 College Street, PO Box 208034, New Haven, CT 06520, USA; [7]Yale School of Medicine, Yale University, 60 College Street, PO Box 208034, New Haven, CT 06520, USA; [8]Department of Gynecology-Oncology, Princess Margaret Hospital, 610 University Avenue, Toronto, ON, Canada M5T 2M9; [9]Department of Obstetrics and Gynecology, Faculty of Medicine, University of Toronto, 610 University Avenue, Toronto, ON, Canada M5T 2M9; [10]Department of Pathology, Princess Margaret Hospital, 610 University Avenue, Toronto, ON, Canada M5T 2M9; [11]Department of Community and Family Medicine, Duke Comprehensive Cancer Center Box 2949, Duke University Medical Center, Durham, NC 27710, USA

BACKGROUND: Mutations in genes for hereditary non-polyposis colorectal cancer (HNPCC) in ovarian cancer patients remains poorly defined. We sought to estimate the frequency and characteristics of HNPCC gene mutations in a population-based sample of women with epithelial ovarian cancer.

METHODS: The analysis included 1893 women with epithelial ovarian cancer ascertained from three population-based studies. Full-germline DNA sequencing of the coding regions was performed on three HNPCC genes, *MLH1*, *MSH2* and *MSH6*. Collection of demographic, clinical and family history information was attempted in all women.

RESULTS: Nine clearly pathogenic mutations were identified, including five in *MSH6*, two each in *MLH1* and *MSH2*. In addition, 28 unique predicted pathogenic missense variants were identified in 55 patients. Pathogenic mutation carriers had an earlier mean age at diagnosis of ovarian cancer, overrepresentation of cancers with non-serous histologies and a higher number of relatives with HNPCC-related cancers.

CONCLUSIONS: Our findings suggest that fewer than 1% of women with ovarian cancer harbour a germline mutation in the HNPCC genes, with overrepresentation of *MSH6* mutations. This represents a lower-range estimate due to the large number of predicted pathogenic variants in which pathogenicity could not definitively be determined. Identification of mismatch repair gene mutations has the potential to impact screening and treatment decisions in these women.

It is estimated that 13% of all invasive ovarian cancer patients carry mutations in *BRCA1* or *BRCA2* (Whittemore *et al*, 1992; Taylor and Schwartz, 1994), and an additional proportion of patients carry mutations in genes that increase risk of hereditary non-polyposis colorectal cancer (HNPCC). Hereditary non-polyposis colorectal cancer is an autosomal dominant syndrome of cancer predisposition of the colorectum, endometrium, stomach or ovaries associated with DNA mismatch repair (MMR) gene mutations. Other less frequent sites include cancers of the renal pelvis, ureter, small bowel, pancreas and brain (Lynch *et al*, 2009). Patients may develop multiple primary cancers, and colorectal cancers are characterised by young age of onset (Vasen, 2005). Hereditary non-polyposis colorectal cancer is believed to account for 2–4% (Hampel *et al*, 2005, 2008) of unselected cases of colorectal cancer, but the fraction of ovarian cancer due to HNPCC is not well established. The lifetime risk of ovarian carcinoma in females carrying HNPCC mutations has been estimated to be up to 12%, with a recent report suggesting cumulative risks of 20% or higher for the *MLH1* and *MSH2* genes; however, risk may vary with the specific gene involved (Bonadona *et al*, 2011).

In most studies of HNPCC families to-date, *MLH1* and *MSH2* account for 80–90% of observed mutations in the MMR genes (Papadopoulos *et al*, 1994; Peltomaki and Vasen, 1997). However, most studies were clinic-based surveys of individuals tested based on clinical criteria, such as the Amsterdam criteria (Vasen *et al*, 1999), and few included *MSH6* testing. Mutations in *MSH6* (Miyaki *et al*, 1997; Sjursen *et al*, 2010) are infrequent in classical HNPCC families, and thus may have been underestimated in prior studies.

Identification of germline MMR genes enables individuals to benefit from up-to-date cancer risk management options as outlined in the NCCN guidelines (Lynch, 2006). In fact, frequent colonoscopy has been shown to improve outcome (Syngal *et al*, 1998; Vasen *et al*, 1998). Furthermore, endometrial and ovarian cancer risk management options include both screening and prophylactic surgery, once childbearing is complete. In addition, preliminary studies in colorectal cancer suggest that knowledge of germline MMR gene mutations may provide an opportunity to refine cancer treatment (Elsaleh *et al*, 2000, 2001; Hemminki *et al*, 2000).

The objective of the current study was to estimate the frequency of mutations in the three HNPCC genes in a population-based sample of women with epithelial ovarian cancer. Secondary objectives included evaluation of demographic, clinical, histopathologic and family history characteristics of germline mutation carriers.

MATERIALS AND METHODS

Participants

Data for this study were drawn from three population-based studies of epithelial ovarian cancer: the Familial Ovarian Tumour Study (FOTS) in Toronto (Risch *et al*, 2001), the Tampa Bay Ovarian Cancer Study (TBOCS) at the Moffitt Cancer Center (Pal *et al*, 2005) and the North Carolina Ovarian Cancer Study (NCOCS) at the Duke University (Wenham *et al*, 2003), with details about study design, populations and data collection methods published previously. Briefly, FOTS cases were identified through monitoring of pathology reports submitted to the population-based Ontario Cancer Registry for province-wide recruitment. The TBOCS cases were recruited through a rapid case ascertainment mechanism in the two most populous counties in the Tampa Bay region. The NCOCS cases were identified through a rapid case ascertainment mechanism in a 48-county region located in the central portion of North Carolina (Wenham *et al*, 2003). The study protocol was approved by the institutional review board at each centre, and written informed consent was obtained from all participants. There was no mechanism of rapid ascertainment in place to recruit the FOTS cases, in contrast to the TBOCS and NCOCS cases, which resulted in a longer time from diagnosis to ascertainment in the Toronto centre compared with the American centres.

Eligibility criteria for study enrolment included diagnosis of incident, pathologically confirmed primary epithelial ovarian cancer, either borderline or invasive, and age 20 years or above. Each study collected questionnaire data concerning demographic, clinical and family history information, and reviewed medical and pathology records for determining tumour histopathology. Specimen collection included blood for DNA extraction and analysis.

Gene sequencing

All 45 coding exons of the *MLH1* (NM_000249.3), *MSH2* (NM_000251.1) and *MSH6* (NM_000179.2) genes were amplified and sequenced in 44 fragments in germline DNA. Primers were designed using Primer 3 software (Rozen and Skaletsky, 2000) to cover at least 20 bp at each 5′- and 3′-side of the exons. The amplified DNA fragments were sequenced by using the BigDye Terminator Cycle Sequencing kit on an ABI 3730xl DNA Analyzer (Applied Biosystems Co., Foster City, CA, USA). Sequencing chromatograms generated by the analyser were examined for variant detection using Mutation Surveyor software (SoftGenetics LLC., State College, PA, USA). All sequences were compared with the related NCBI (http://www.ncbi.nlm.nih.gov) reference sequences for variant detection. The chromatograms of all the computationally determined variants were confirmed manually.

All insertions and deletions in the gene-coding regions, nonsense variants and variants located at the essential splice site sequences were considered as potentially pathogenic, and their DNA fragments were also sequenced in the reverse direction for confirmation. Insertions and deletions for which no functionality data were available were classified as 'unknown pathogenicity'. Missense variants first were searched in the colon cancer gene variant database (http://chromium.liacs.nl/LOVD2/colon_cancer/home.php) for functional effects. If functional effect was not known, we used Align-GVGD (Tavtigian *et al*, 2006) to predict the likely functional effect based on library alignments from human to pufferfish for *MLH1* and *MSH2*. As no alignment data were available for *MSH6* in the Align-GVGD website, a protein alignment was constructed for predicting the functional effect of *MSH6* missense variants from *MSH6* homologues in eight species (human, chimpanzee, dog, cow, pig, mouse, chicken, frog and zebrafish). Missense variants with class C45 and higher defined by Align-GVGD were classified as 'predicted pathogenic'. The class C45 was chosen as a cut-off point for Align-GVGD based on receiver-operating characteristic curve analysis using *BRCA1* and *BRCA2* missense variants with known functional effects reported in the Breast Cancer Information Core database (http://research.nhgri.nih.gov/projects/bic/) or Myriad's published data (Easton *et al*, 2007). The rationale for using *BRCA1* and *BRCA2* mutations for the receiver-operating characteristic curve analysis was the limited sample size of known pathogenic missense mutations in the *MLH1*, *MSH2* and *MSH6* genes. The chosen cut-off point corresponds to 90% specificity and 50% sensitivity. We also used PolyPhen2 (Adzhubei *et al*, 2010) and SIFT (Ng and Henikoff, 2003) for predicting the functional effect of missense variants. We considered a missense variant as a predicted pathogenic if two of the three *in silico* tools (Align-GVGD, PolyPhen-2 and SIFT) predicted it as pathogenic and the carrier frequency of that mutation was less than 1% among 6481 study subjects of the NHLBI Exome Sequencing Project (ESP6500) (http://evs.gs.washington.edu/EVS/).

Data collection

All participants completed questionnaires by which demographic and family history information was obtained. Family history data included types and ages of cancer diagnoses in first-, second- and third-degree relatives. In addition, the FOTS data set included ages of relatives (current, or if deceased, age at death). Medical records were retrieved on all participants to abstract information on tumour histology. Information on date of diagnosis (based on pathology report) and date of study enrolment (based on date consent form was signed) were collected, to determine time between diagnosis and enrolment (calculated in days).

Statistical analyses

Site differences in descriptive statistics of clinical factors across the three sites were evaluated using analysis of variance and Kruskal–Wallis tests for continuous variables (e.g., time to diagnosis and attained age) and Pearson's χ^2-tests for categorical variables. Similarly, clinical variables were compared across mutation subtypes (i.e., pathogenic, predicted pathogenic, predicted non-pathogenic, no mutation detected). Known benign polymorphic variants were combined with the 'no mutation' group. All reported *P*-values are two-sided. All analyses were carried out with SAS version 9.1.3 (SAS Institute, Inc., Cary, NC, USA).

To compare the cumulative incidence of cancer among the first-degree relatives of proband carriers of different mutation subtypes, a survival analysis approach among the FOTS study subjects for whom we had detailed family history information was used. Each first-degree relative was considered as an observation and followed from birth until the occurrence of any type of cancer, death from

another cause, or the date of the study interview, using the Cox proportional hazard model. Separate analyses were carried out for all cancer outcomes, all HNPCC cancer outcomes, colorectal cancer outcome and ovarian cancer outcome. Covariates for each observation included the proband's mutation status. Hazard ratios of mutation subtypes and 95% confidence interval (CI) were estimated for each model.

RESULTS

In total, 1893 ovarian cancer patients were included, with 1521 from FOTS, 126 from TBOCS and 246 from NCOCS, as shown in Table 1. Overall, mean age at diagnosis and racial distribution were similar between sites, other than a higher proportion of Black subjects in the Duke sample. The median time between diagnosis and study enrolment was similar between the Duke and Moffitt cases, although longer for the Toronto cases because of the difference in participant ascertainment procedure. At all three sites, the majority of cancers were of serous histology. However, the proportion of non-serous cases was higher in the Ontario case than in the other two sites. Family histories of HNPCC-associated cancers were similar across the three sites.

Germline genetic testing of coding regions in the *MLH1*, *MSH2* and *MSH6* genes revealed sequence changes at 161 different nucleotides. As shown in Table 2, nine pathogenic mutations were found, five in *MSH6*, two in *MLH1* and two in *MSH2*. Four mutations were insertions or deletions and five were nonsense mutations. Seven of the nine pathologic mutations have been previously reported and each of the nine was seen in a single individual. We detected two additional changes of 'unknown pathogenicity' in *MSH6*: an in-frame deletion of two amino acids (*MSH6* c.936_941delGAAAAG), not known to belong to any known functional protein domain, and a frame-shift insertion (*MSH6* c.4065_4066insGTGA) at the C terminus that truncates two terminal amino acids. In addition, there were 101 missense variants (Supplementary Table 1), including 5 benign polymorphisms, and 96 unclassified variants (in 128 participants) and 49 silent variants (Supplementary Table 2). Overall, the frequency of pathogenic mutations was 0.5% (95% CI 0.2–0.8; Table 2). However, this frequency was 1.6% (95% CI 0.4–2.7) when considering only invasive cancers of the endometrioid and clear cell subtypes.

Through the Align-GVGD, PolyPhen and SIFT algorithms, pathogenicity was evaluated in the 96 unclassified variants, by which 28 unclassified variants (in 55 participants) were classified as 'predicted pathogenic' and 68 (in 99 participants) as 'predicted non-pathogenic'. To further evaluate our predictions of pathogenicity, we compared the penetrance among 9015 first-degree relatives of 1521 FOTS participants by mutation subtype. Results showed that cumulative risk to age 80 years of any cancer in those with pathogenic mutations was 56.7% compared with 44.2, 21.0 and 27.4% for those in the 'predicted pathogenic', predicted 'non-pathogenic', and no mutations groups, respectively. A similar result was seen for the risk of developing HNPCC-related cancer, but the difference between the groups did not reach customary statistical significance (Table 3). Cumulative risk of ovarian cancer among relatives of mutation carriers compared with relatives of non-carriers was not elevated, likely due to the limited number of relatives with ovarian cancer.

The average age of onset of ovarian cancer was 47.1 years in the 'pathogenic group' (Table 4) and 53.2 years in the 'predicted pathogenic group', compared with 56.1 years in those with no mutations. Non-serous cancers comprised 77.8% in the 'pathogenic group' and 66% in the 'predicted pathogenic group', compared with 41.9% in those with no mutations. The number of relatives with HNPCC-related cancers was highest in the 'pathogenic' group; however, findings in the other three groups indicated similar proportions. Of note, the proportion of mutations in the four mutation subgroups was similar across study sites ($P = 0.2$). Specifically, the proportion of mutation carriers in the Moffitt, Duke and Toronto samples was 0.8%, 0.4% and 0.5%, respectively, despite the longer time from diagnosis to enrolment in the Toronto cases.

Table 1 Demographic and clinical characteristics of ovarian cancer patients by study site

	Overall (N = 1893)	Duke (N = 246)	Moffitt (N = 126)	Toronto (N = 1521)	P-value
Mean age (s.d.)	56.0 (12.2)	55.2 (11.3)	56.7 (11.6)	56.1 (12.4)	0.45
Race, n (%)					
Caucasian	1751 (92.5)	213 (86.6)	121 (96.0)	1417 (93.2)	<0.0001
Black	38 (2.0)	28 (11.4)	2 (1.6)	8 (0.5)	
Asian	94 (5.0)	2 (0.8)	3 (2.4)	89 (5.9)	
Other	10 (0.5)	3 (1.2)	0 (0)	7 (0.5)	
Time from diagnosis to interview, days (median)	613	118	108	677	<0.0001
Histology					
Borderline, n (%)	255 (13.5)	51 (20.7)	15 (11.9)	189 (12.4)	0.0019
Invasive, n (%)	1638 (86.5)	195 (79.3)	111 (88.1)	1332 (87.6)	
Serous, n (%)	933 (57.0)	130 (66.7)	66 (59.5)	737 (55.3)	0.0099
Non-serous, n (%)	705 (43.0)	65 (33.3)	45 (40.5)	595 (44.7)	
Clear cell	116 (7.1)	15 (7.7)	8 (7.2)	93 (7.0)	0.17
Endometrioid	313 (19.1)	20 (10.3)	19 (17.1)	274 (20.6)	
Mucinous	127 (7.8)	9 (4.6)	5 (4.5)	113 (8.5)	
Other[a]	149 (9.1)	21 (10.8)	13 (11.7)	115 (8.6)	
Family history, n (%)[b]					
% of subjects with relatives with colorectal cancer	363 (19.2)	34 (13.8)	20 (15.9)	309 (20.3)	0.037
% of subjects with relatives with endometrial cancer	59 (3.1)	11 (4.5)	8 (6.3)	40 (2.6)	0.031
% of subjects with relatives with any HNPCC cancer[c]	637 (33.7)	80 (32.5)	38 (30.2)	519 (34.1)	0.61

Abbreviation: HNPCC = hereditary non-polyposis colorectal cancer. [a]Other includes the following histologies: carcinoma, unspecified (108), mixed cell (34), ovarian surface epithelial tumour (1), primary peritoneal (1) and transitional cell carcinoma (5). [b]Family history used the following relatives: mother, father, sisters, brothers, daughters, sons, grandmother, grandfather, aunt, uncle, nieces, nephews, halfsiblings. [c]The HNPCC cancer sites included colorectum, endometrium, other gastrointestinal tract, urinary tract, ovary and brain.

Genetics and Genomics

Table 2 Summary of pathogenic, predicted pathogenic and unknown variants in the MMR genes

	Gene	Mutation	Protein change	Mutation type	Functional effect	GVGD prediction	PolyPhen prediction	SIFT prediction	ESP6500 carrier frequency	No. of individuals with mutation
1	MLH1	c.676C>T	p.Arg226X	N	P	NA	NA	NA	0	1
2	MLH1	c.1852_1854delAAG	p.Lys618del	ID	P	NA	NA	NA	0	1
3	MSH2	c.163delC	p.Arg55GlyfsX9	FD	P	NA	NA	NA	0	1
4	MSH2	c.2038C>T	p.Arg680X	N	P	NA	NA	NA	0	1
5	MSH6	c.1636G>T	P.Arg312_Lys313del	N	P	NA	NA	NA	0	1
6	MSH6	c.2150_2153delTCAG	p.Glu546X	FD	P	NA	NA	NA	0	1
7	MSH6	c.2690_2691insA	p.Val717fsX18	FI	P	NA	NA	NA	0	1
8	MSH6	c.2731C>T	p.Asn897fsX3	N	P	NA	NA	NA	1	1
9	MSH6	c.3103C>T	p.Arg911X	N	P	NA	NA	NA	0	1
10	MSH6	c.936_941delGAAAAG	p.Arg1035X	ID	U	NA	NA	NA	0	1
11	MSH6	c.4065_4066insGTGA	p.Leu1356fsX4	FI	U	NA	NA	NA	0	1
12	MLH1	c.47T>G	p.Val16Gly	M	PP	Class C65	PrD	D	0	1
13	MLH1	c.1136A>G	p.Tyr379Cys	M	PP	Class C25	PoD	D	2	1
14	MLH1	c.1148T>C	p.Met383Thr	M	PP	Class C65	PrD	D	2	1
15	MLH1	c.1151T>A	p.Val384Asp	M	PP	Class C65	PrD	D	1	3
16	MLH1	c.1489C>G	p.Arg497Gly	M	PP	Class C45	PoD	T	0	1
17	MLH1	c.1808C>G	p.Pro603Arg	M	PP	Class C65	PoD	D	1	1
18	MLH1	c.1852_1853delAAinsGC	p.Lys618Ala	M	PP	Class C65	PrD	D	0	24
19	MLH1	c.1870G>C	p.Asp624His	M	PP	Class C0	PrD	D	0	1
20	MLH1	c.1937A>G	p.Tyr646Cys	M	PP	Class C65	PrD	D	0	1
21	MSH2	c.1A>C	p.Met1Leu	M	PP	Class C0	PrD	D	0	1
22	MSH2	c.138C>G	p.His46Gln	M	PP	Class C15	PrD	D	3	1
23	MSH2	c.166G>A	p.Glu56Lys	M	PP	Class C55	PrD	T	0	1
24	MSH2	c.1044G>C	p.Gln348His	M	PP	Class C15	PrD	D	0	1
25	MSH2	c.1432C>T	p.Leu478Phe	M	PP	Class C15	PrD	D	0	1
26	MSH2	c.1927G>A	p.Glu643Lys	M	PP	Class C55	PrD	D	1	1
27	MSH2	c.2203A>G	p.Ile735Val	M	PP	Class C25	PoD	D	3	1
28	MSH2	c.2542G>T	p.Ala848Ser	M	PP	Class C65	PoD	D	0	1
29	MSH2	c.2558A>G	p.Glu853Gly	M	PP	Class C65	PoD	D	0	1
30	MSH2	c.2732T>G	p.Leu911Arg	M	PP	Class C45	PrD	D	0	1
31	MSH2	c.437G>C	p.Arg146Thr	M	PP	Class C25	PrD	D	0	1
32	MSH2	c.802G>C	p.Asp268His	M	PP	Class C0	PrD	D	0	1
33	MSH6	c.2561A>T	p.Lys854Met	M	PP	Class C65	PrD	D	5	1
34	MSH6	c.2594T>G	p.Phe865Cys	M	PP	Class C65	PoD	T	0	1
35	MSH6	c.2752C>T	p.His918Tyr	M	PP	Class C65	PrD	T	0	1
36	MSH6	c.3259C>T	p.Pro1087Ser	M	PP	Class C65	PoD	T	4	1
37	MSH6	c.3407A>G	p.Asn1136Ser	M	PP	Class C45	PrD	D	0	2
38	MSH6	c.3722G>A	p.Cys1241Tyr	M	PP	Class C65	PrD	D	0	1
39	MSH6	c.3938T>C	p.Ile1313Thr	M	PP	Class C65	PoD	T	0	2

Abbreviations: D = damaging; FD = frameshift deletion; FI = frameshift insertion; GVGD = Grantham Variation and Grantham Deviation; ID = in-frame deletion; M = missense; MMR = mismatch repair; N = nonsense; P = pathogenic; PoD = possibly damaging; PP = predicted pathogenic; PrD = probably damaging; T = tolerated; U = unknown.

Finally, review of family histories of those with pathogenic and predicted pathogenic mutations indicated the two families that met clinical criteria for HNPCC (Vasen et al, 1999) carried pathogenic MLH1 mutations. Furthermore, the two families with pathogenic MSH2 mutations had striking family histories, despite not meeting clinical criteria. All remaining families did not meet clinical criteria.

DISCUSSION

We determined the frequency of germline mutations in three MMR genes in a population-based sample of 1893 women with ovarian cancer. Our findings suggest that pathogenic germline MMR mutations are found in less than 1% of unselected ovarian cancer cases, with a higher frequency in those with invasive cancers of the endometrioid and clear cell subtypes. Of the nine pathogenic mutations clearly identified, the majority (55%) were detected in the MSH6 gene.

The pathogenic mutations clearly identified in our study were classified based on protein truncation or on previous epidemiologic or functional studies. Our findings likely represent an underestimate of the true proportion, as there were several missense variants for which pathogenicity could not be definitively determined. Furthermore, we did not test for additional less common MMR genes (PMS2, EPCAM) or for large gene rearrangements, which may have detected additional pathogenic mutations (de Jong et al, 2004; Grabowski et al, 2005; van der Klift et al, 2005; Gylling et al, 2009; Kovacs et al, 2009).

For the many missense variants detected in the study, databases such as UniProt (www.uniprot.org) and Human Genome Mutation Database (www.hgmd.org) were explored to determine pathogenicity. Of note, UniProt, a protein database, had many errors in functional classification of missense variants and, thus, is unreliable for definitively determining the functional effect of individual variants. Similarly, the Human Genome Mutation Database, a manually curated database, classifies many missense mutations as disease-causing without providing compelling evidence from the literature to support the assignments. Consequently, we focused on bioinformatic tools to establish the pathogenicity of missense variants (Tavtigian et al, 2008). Specifically, we used the Align-GVGD (Tavtigian et al, 2006) algorithm by means of a manually curated protein alignment across species of broad phylogenic scope in combination with a stringent cut-off point to predict the dysfunctional effect of a variant. This method outperforms other bioinformatic tools

Table 3 Penetrance of cancer among 9015 first degree relatives of 1521 FOTS participants by mutation subtype

Cohort groups of first-degree relatives	Affected relatives/ total relatives	Cumulative risk to age 80, % (s.e.)	Hazard ratio	95% Confidence interval	P-value
All cancers					
No mutation	876/7905	27.4 (0.9)	Reference		
Pathogenic mutation	8/33	56.7 (15.2)	4.72	2.33–9.56	<0.0001
Unclassified variant	73/640	28.7 (3.4)	1.09	0.86–1.38	0.5
Predicted pathogenic	35/205	44.2 (6.7)	1.75	1.24–2.45	0.001
Predicted non-pathogenic	38/435	21.0 (3.4)	0.81	0.58–1.12	0.20
HNPCC-related cancers[a]					
No mutation	345/7890	13.0 (2.7)	Reference		
Pathogenic mutation	4/33	28.8 (13.5)	6.12	2.26–16.5	0.0004
Unclassified variant	25/626	11.9 (2.6)	0.93	0.62–1.40	0.73
Predicted pathogenic	11/202	17.1 (5.7)	1.33	0.73–243	0.35
Predicted non-pathogenic	14/424	9.0 (2.8)	0.76	0.44–1.29	0.31
Colorectal cancer					
No mutation	120/8023	5.3 (0.5)	Reference		
Pathogenic mutation	3/33	16.9 (9.2)	15.5	4.88–49.5	<0.0001
Unclassified variant	9/653	5.5 (2.1)	0.96	0.49–1.89	0.9
Predicted pathogenic	3/2114	8.2 (0.5)	1.05	0.33–3.29	0.94
Predicted non-pathogenic	6/442	4.3 (2.2)	0.92	0.41–2.09	0.84
Ovarian cancer[b]					
No mutation	67/4166	3.6 (0.5)	Reference		
Pathogenic mutation	0/16	0 (0.0)	0	Not applicable	0.99
Unclassified variant	6/325	3.4 (1.5)	1.16	0.50–2.67	0.73
Predicted pathogenic	3/104	4.3 (2.5)	2.00	0.63–6.37	0.24
Predicted non-pathogenic	3/221	3.1 (1.8)	0.81	0.26–2.59	0.72

Abbreviations: FOTS = Familial Ovarian Tumour Study; HNPCC = hereditary non-polyposis colorectal cancer. Note the following categories of genetic test results: unclassified variants, missense variants with uncertain functional effect; predicted pathogenic, predicted based on Align-Grantham Variation and Grantham Deviation. [a]The HNPCC cancer sites included colorectum, endometrium, other gastrointestinal tract, urinary tract, ovary and brain. [b]Female first-degree relatives of the probands were included in this analysis.

(Akbari *et al*, 2011) such as SIFT (Ng and Henikoff, 2003) and PolyPhen2 (Adzhubei *et al*, 2010). However, we used the combination of these three tools for predicting the functional effect of the missense variants. We also looked at the carrier frequency of these variants in ESP6500 database and considered the frequently reported ones as predicted 'non-pathogenic'. We detected 28 missense mutations (in 55 participants) classified as 'predicted pathogenic' using these tools. Thus, if all of these are in fact pathogenic, the prevalence of MMR gene mutation carriers among ovarian cancer patients would be 3.6% or higher. Of note, we chose not use the MAPP-MMR tool (Chao *et al*, 2008; which is another bioinformatics tool with no clear advantage to other tools), because it only predicts the functional effects in *MLH1* and *MSH2* genes, whereas over 40% of the missense variants identified in our sample were in *MSH6*. Furthermore, because of conflicting reports in the literature regarding pathogenicity of certain variants (e.g., *MLH1* p.Lys618A1a, observed in 24 participants in our study, was previously reported as both non-pathogenic (Raevaara *et al*, 2005) and pathogenic; Pastrello *et al*, 2011), we reported predictions of pathogenicity according to aforementioned criteria rather than based on prior published reports, which are in essence based on predictions.

Prior estimates of the frequency of germline MMR mutations in ovarian cancer patients have been less than 5% (Rubin *et al*, 1998; Stratton *et al*, 1999), based on studies of 116 patients or fewer and limited to the *MLH1* and *MSH2* genes only. Moreover, one of the studies was restricted to women aged 30 years or less at diagnosis (Stratton *et al*, 1999). Thus, although our prevalence estimates are generally lower than prior reports, the validity and generalisability of our data is enhanced by the much larger sample size, performance of testing in all three genes (i.e., including *MSH6*) and inclusion criteria that encompassed a wider age group of participants.

Although most studies have focused on *MLH1* and *MSH2*, several have reported ovarian cancer in the *MSH6* tumour spectrum (Miyaki *et al*, 1997; Wu *et al*, 1999; Wagner *et al*, 2001; Bonadona *et al*, 2011). However, in contrast to prior reports suggesting only 10% of HNPCC families harbour *MSH6* mutations (Wijnen *et al*, 1999; Berends *et al*, 2002), such mutations accounted for over half of the clearly pathogenic mutations in our study. The low percentage of *MSH6* mutations may be due to inadequate clinical criteria to accurately identify these families (Sjursen *et al*, 2010), who typically present with later age of colorectal cancer, and higher risk but later age of endometrial cancer in females (Wijnen *et al*, 1999; Wagner *et al*, 2001; Devlin *et al*, 2008; Ramsoekh *et al*, 2009). Thus, although infrequent in classical HNPCC families, our results suggest *MSH6* mutations may be better represented in families with ovarian cancer. In fact, a recent study of 67 *MLH1*, *MSH2* and *MSH6* mutation carriers ascertained through a family cancer clinic included 10 ovarian cancers, of which the majority were seen in *MSH6* carriers (Ramsoekh *et al*, 2009), consistent with our study findings. Interestingly, none of the families with pathogenic *MSH6* mutations in our study met clinical criteria for HNPCC (Vasen *et al*, 1999). In contrast, both *MLH1* families met clinical criteria, and both *MSH2* families had striking family histories, despite not meeting clinical criteria. Of note, clinical criteria do not include cancers of the ovary and stomach, yet these cancers are much more common in HNPCC compared with those of the small bowel, ureter and renal pelvis, which are included. Ultimately, our study underscores the limitations of the use of HNPCC clinical diagnostic criteria based only on personal and family cancer history to identify *MSH6* families.

Our results were consistent with prior reports, suggesting an earlier age at diagnosis of ovarian cancer in carriers (mean ranging from 41 to 49 years) compared with sporadic cases (mean ranging from 60 to 65 years) (Watson and Lynch, 2001; Crijnen *et al*, 2005;

Genetics and Genomics

Table 4 Mutation subtype by demographic, clinical, histopathologic and family history variables

	Pathogenic (n = 9)	Predicted pathogenic (n = 61)	Predicted non-pathogenic (n = 91)	No mutation detected (n = 1730)	P-value
Mean age (s.d.)	47.1 (6.0)	53.2 (15.2)	57.1 (11.9)	56.1 (12.1)	0.032
Age ⩽50, n (%)	7 (77.8)	26 (42.6)	31 (34.1)	563 (32.5)	0.012
Age >50, n (%)	2 (22.2)	35 (57.4)	60 (65.9)	1167 (67.5)	
Race, n (%)					
Caucasian	8 (88.9)	56 (93.4)	75 (82.4)	1610 (93.1)	
Black	1 (11.1)	1 (1.6)	3 (3.3)	32 (1.8)	0.056
Asian	0 (0)	3 (5.0)	13 (14.3)	78 (4.5)	
Other	0 (0)	0 (0)	0 (0)	10 (0.6)	
Time from diagnosis to interview, days (median)	784	576	671	610.5	0.271
Histology					
Borderline, n (%)	0 (0)	11 (18.0)	6 (6.6)	237 (13.7)	0.083
Invasive, n (%)	9 (100)	50 (82.0)	85 (93.4)	1493 (86.3)	
Serous, n (%)	2 (22.2)	17 (34.0)	45 (52.9)	868 (58.1)	0.001
Non-serous, n (%)	7 (77.8)	33 (66.0)	40 (57.1)	625 (41.9)	
Clear cell	2 (28.6)	6 (18.2)	8 (20.0)	100 (16)	
Endometrioid	5 (71.4)	16 (48.5)	20 (50.0)	272 (43.5)	0.801
Mucinous	0 (0)	5 (15.2)	5 (12.5)	117 (18.7)	
Other[a]	0 (0)	6 (18.2)	7 (17.5)	136 (21.8)	
Family history[b]					
Relatives with colorectal cancer, n (%)	4 (44.4)	8 (13.3)	16 (17.4)	335 (19.4)	0.143
Relatives with endometrial cancer, n (%)	0 (0)	1 (1.7)	4 (4.3)	54 (3.1)	0.731
Relatives with any HNPCC cancer[c], n (%)	4 (44.4)	18 (30)	31 (33.7)	584 (33.8)	0.832
Number of mutations					
Toronto, n (proportion)	7 (0.5)	41 (2.7)	76 (5.0)	1396 (91.8)	
Tampa, n (proportion)	1 (0.8)	6 (4.8)	5 (4.0)	114 (90.5)	0.244
Duke, n (proportion)	1 (0.4)	14 (5.7)	10 (4.1)	220 (89.8)	

Abbreviations: HNPCC = hereditary non-polyposis colorectal cancer. [a]Other includes the following histologies: carcinoma, unspecified (108), mixed cell (34), ovarian surface epithelial tumour (1), primary peritoneal (1) and transitional cell carcinoma (5). [b]Family history used the following relatives: mother, father, sisters, brothers, daughters, sons, grandmother, grandfather, aunt, uncle, nieces, nephews, halfsiblings. [c]The HNPCC cancer sites included colorectum, endometrium, other gastrointestinal tract, urinary tract, ovary and brain.

Malander *et al*, 2006). Moreover, our age of diagnosis ranged from 40 to 59 years, which suggests that prophylactic surgery offered before age 40 years is likely to prevent the majority of HNPCC-associated ovarian cancers. Furthermore, the overrepresentation of non-serous tumours, particularly of endometrioid and clear cell subtypes (Pal *et al*, 2008a, b; Ketabi *et al*, 2011), reported in HNPCC is consistent with our findings. Finally, as in our prior studies (Akbari *et al*, 2011), we used a cohort analysis to evaluate the Align-GVGD predictions. For example, if the predicted pathogenic mutations have the same penetrance, on average as the clearly pathogenic mutations, then we would expect to see cancer risks in first-degree relatives of patients in the two subgroups to be similar. In our study, although the penetrance of predicted pathogenic variants was slightly lower than the pathogenic variants, it was still consistently higher than the 'predicted non-pathogenic' and 'no mutation' groups, thus suggesting that many in this class are likely pathogenic. A deviation from this trend was seen in the lifetime risk of ovarian cancer illustrated in Table 3, which was lowest in the 'pathogenic mutation' group; however, this may be a spurious finding due to the limited number of relatives with ovarian cancer. Overall, the combined frequency of pathogenic and predicted pathogenic mutations was 3.6% (95% CI 2.9–4.6; Table 2). However, this frequency was 6.3% (95% CI 4.3–9.2) when considering only invasive cancers of the endometrioid and clear cell subtypes.

A number of strengths support the current study, including the large sample size, the population-based study design and comprehensive collection of clinical and demographic data. Despite these strengths, there remain some limitations, including

our testing strategy that may have led to underestimating mutation frequency. Specifically, because of limited resources, large rearrangement testing was not performed, which may account for 10–20% of mutations in the MMR genes (Grabowski *et al*, 2005; van der Klift *et al*, 2005; Gylling *et al*, 2009). Similarly, testing for the two additional MMR genes, which has recently become available (i.e., *PMS2* (de Jong *et al*, 2004) and *EPCAM* (Kovacs *et al*, 2009)) was also not performed in the current study. In addition, differences in demographic and clinical variables across sites (as reported in Table 1) could potentially affect our results. Notably, the time to diagnosis in FOTS cases was significantly longer than that for TBOCS or NCOCS, leading to overrepresentation of cases with longer survival in the FOTS data set. However, the main concern here is whether a longer time to diagnosis led to overrepresentation of mutation-positive cases in the FOTS data set, thereby biasing our mutation prevalence estimate. If this were true, the proportion of mutation-positive cases in the FOTS sample would be higher, which was not the case (as illustrated in Table 4). Therefore, it appears the longer time to diagnosis in the FOTS data set did not appear to substantially affect the mutation prevalence estimate. Nevertheless, the presence of survival bias within the FOTS data may have skewed the clinical characteristics within the overall data set as suggested by the higher proportions of non-serous cases observed. However, there remained an association between non-serous histologies and HNPCC mutations, which was likely underestimated because of the lower numbers of serous cases within the data set. Finally, we were limited in our ability to predict pathogenicity in many of the missense mutations because of sparse data available in the literature and mutation databases.

Genetics and Genomics

To address this, we used an *in silico* approach to predict the dysfunctional effect of the detected missense variants, recognising that inherent inaccuracy of bioinformatics tools could result in some misclassification of the variants. However, our findings suggest that many are likely to be pathogenic based on similarities in the clinical and demographic characteristics with the pathogenic group (as shown in Tables 3 and 4).

In summary, we estimate that less than 1% of unselected ovarian cancer patients may have mutations in the MMR genes, with overrepresentation of *MSH6* mutations in those with invasive cancers of the endometrioid and clear cell subtypes. Ovarian tumours with MMR-deficiency are characterised by early age of onset and overrepresentation of non-serous cancers. Our results suggest that current clinical criteria for HNPCC are insufficient to identify the majority of ovarian cancer patients with mutations.

These findings highlight the need to consider HNPCC testing in women with ovarian cancer, particularly those with invasive cancers of the endometrioid and clear cell subtypes, even if they do not meet strict clinical criteria for the condition.

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