

Published in final edited form as:

Gynecol Oncol. 2008 June ; 109(3): 384–387. doi:10.1016/j.ygyno.2007.11.046.

Ovarian cancer risk is associated with a common variant in the promoter sequence of the mismatch repair gene *MLH1*

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Abstract

Objectives—Inherited mutations in the *MLH1* gene are associated with a proportion of families with the hereditary non-polyposis colon cancer syndrome (HNPCC). The cardinal features of the syndrome are a predisposition to colon, endometrial and ovarian cancers. Recently, it has been shown that a non-coding polymorphic variant in *MLH1* (G>A nt-93) predisposes to colon and endometrial cancer, but with much reduced penetrance. We sought to establish whether or not this polymorphic variant also predisposes to ovarian cancer.

Methods—We genotyped 899 women with invasive ovarian cancer and 931 controls for the G>A nt-93 variant.

Results—The presence of the variant was associated with a modest, but highly significant risk of ovarian cancer (OR = 1.5; 95% CI 1.3–1.9; $p = 0.00005$). The association was present in cancers of all histologies except clear cell, and in all ethnic groups.

Conclusions—The G>A nt-93 variant of the *MLH1* gene is associated with an increased risk of invasive ovarian cancer.

Keywords

Ovarian cancer; *MLH1*; Association; Mismatch repair

Introduction

Ovarian cancer is the leading cause of death from gynecological malignancy in North America. The lifetime risk of ovarian cancer in women from North America is approximately 1.5% [1]. Genetic factors are important in the development of epithelial

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Conflict of interest statement No conflict of interest.

ovarian cancer — approximately 12% of all invasive cancers are due to the inheritance of a BRCA1 or BRCA2 mutation [2]. Mutations in the mismatch repair (MMR) genes *MSH2*, *MLH1* and *MSH6* are much less common in epithelial ovarian cancer, and account for approximately 1–2% of cases [3,4]. MMR genes are responsible for post-replicative genome surveillance. They code for proteins that correct mismatches generated during DNA replication and escape proofreading [5,6]. The importance of the MMR genes in protecting cells from oncogenic progression is illustrated by the role they play in hereditary cancer syndromes [7,8]. Hereditary Non-Polyposis Colorectal Cancer (HNPCC) is an inherited predisposition to colon, endometrial and ovarian cancer, and is due to germ-line mutations in the mismatch repair genes *MSH2*, *MLH1* and *MSH6*. Women with a mutation in one of the HNPCC-related MMR genes have a 10% risk of developing ovarian cancer by the age of 80 years [9].

The hallmark of MMR gene mutations is an alteration in the length of repetitive DNA microsatellite sequences and resulting genomic instability (microsatellite instability — MSI). The frequency of MSI in epithelial ovarian cancer is not well known, but has reported to be as high as 37% [10]. MSI is present in a greater number of ovarian cancers than can be accounted for by inherited MMR mutations alone (1–2%). This suggests that alternative mechanisms are responsible for defective MMR in epithelial ovarian cancers, including common polymorphic variation. Two recent studies have identified a single nucleotide polymorphism (SNP) in the promoter region (nt-93) of *MLH1* to be associated with an increased risk of endometrial and colorectal cancer. Beiner et al. found that carriers of the *MLH1* nt-93 rare A allele were associated with a 1.5-fold increased risk of developing endometrial cancer when compared with controls ($p = 0.001$) [11]. The risk was even higher in women who were homozygotes of the rare A allele (OR 1.9, $p = 0.009$). In the second study, Raptis et al. found an association between carriers of the rare A allele of *MLH1* and colorectal cancer [12]. The increased cancer risk was restricted to patients who had MSI-high colon cancer — with odds ratios of 1.8 and 3.2 for heterozygotes and homozygotes, respectively. We report the results of a large population-based association study assessing the relationship between the *MLH1* nt-93 promoter polymorphism and susceptibility to ovarian cancer.

Materials and methods

Study population

This work is part of an ongoing Ontario-wide population-based genetic epidemiologic study of ovarian cancer [2]. Cases were ascertained through the Ontario Cancer Registry. All women newly diagnosed with invasive epithelial ovarian cancer in Ontario, Canada, from January 1995 to December 1999 were eligible. Of 1694 potentially eligible cases, 1016 women consented and provided blood samples for DNA testing. There were 899 women for whom both DNA and sufficient clinical information were available and these are the subjects of the current study.

We included 1063 controls; these women were selected from healthy women who attended an outpatient screening clinic for well-women at the Women's College Hospital, Toronto, between 1996 and 2001. The controls were invited to participate in a general study by a member of the research team at the time of the outpatient appointment. The clinic offers general preventive health care services to unselected women, including blood lipid and blood pressure monitoring, bone densitometry and cervical and breast screening (mammography). The clinic does not focus on cancer prevention or offer genetics services. Women with a past history of cancer were excluded as controls. Of the women who were approached in the clinic to participate in this study, 80% agreed and provided a blood sample and completed a risk factor questionnaire. The risk factor questionnaire included

questions about medical histories and past and current hormonal exposures, but did not include questions regarding family histories of cancer. DNA samples were no longer available for 132 controls and these were excluded, reducing the total number of controls to 931.

Study subjects were asked to provide details about their ethnic origin, including information about the place of birth of their four grandparents. All study subjects provided informed consent for genetic testing. On average cases were diagnosed at 57.1 years of age. The mean age of the controls was 56.7 years at the time of blood draw.

DNA was extracted from lymphocytes of cases and controls. SNP genotyping was performed using the MassARRAY system (Sequenom Inc., San Diego, CA). Assays were designed using Assay Design 2.0 (Sequenom®). Following PCR amplification, SAP treatment and extension reactions, products were analyzed by Chip-based MALDI-TOF mass spectrometry. Extension primers were designed to extend beyond the SNP site by one or two bases. The choice of the reverse or forward extension primer was based on GC content, primer dimerisation, hairpin structures and the presence of any additional SNP site. The extension reaction mixture contained the following nucleotides: dideoxy ACT and deoxy G.

Primer extension and PCR were performed according to the manufacturer's standard protocol (homogenous MassEXTEND, Sequenom Inc., San Diego, CA). PCR reaction volume was 5 µl including dNTPs, primers, HotStar Taq Polymerase (Qiagen GmbH, Hilden, Germany). The total amount of genomic DNA was 4.0 ng per reaction. After denaturation at 95 °C for 15 min, samples were subjected to 45 cycles of denaturation at 95 °C (20 s), annealing at 56 °C (30 s) and elongation at 72 °C (60 s) with the final cycle at 75 °C for 3 min. Following PCR the residual unincorporated dNTPs were removed by incubating the samples with shrimp alkaline phosphatase (SAP) at 37 °C (20 min). Allele specific primer extension reaction was conducted by adding to each sample appropriate deoxy and dideoxy nucleotide (4.5 nmol), extension primer (10 nmol), thermosequence (0.64 U) with the final volume of 9 µl per reaction. Allele specific extension products were obtained under the following thermocycling conditions: 95 °C for 2 min, followed by 75 cycles of 94 °C for 5 s, 52 °C for 5 s and 72 °C for 5 s. After desalting of the samples with SpectroCLEAN (Sequenom Inc., San Diego, CA) analysis was performed in fully automated mode with the MALDI-TOF MassARRAY system (Bruker-Sequenom, San Diego, CA). Genotype calling was completed in the automated mode by SpectroCALLER (Sequenom Inc., San Diego, CA). For 212 individuals it was not possible to establish the genotype with certainty (11.5%) and these were excluded from the study for the construction of the odds ratios. A representative proportion of the remaining genotypes were verified by direct sequencing and all were concordant.

Statistical analyses

Cases and controls were stratified by ethnic group: White non-French–Canadian (subsequently called “other white”), French–Canadian, other/mixed, and unknown. Odds ratios (OR) were used to estimate the relative risk of ovarian cancer. The data on the entire control group was used to estimate the allele frequencies for generating the odds ratio for subgroups of cases based on histology, age and family history. It is not meaningful to categorize controls by histologic subtype and we did not subdivide the control data on age because the prevalence of the alleles in the population is not expected to vary by age. A positive family history was defined as one or more first- or second-degree relatives with breast or ovarian cancer. We did not have detailed data on cancer family histories of controls. However, allele frequencies may vary by ethnic group and therefore the controls were subdivided by ethnicity for these subgroup comparisons. Mantel–Haenszel procedures

were employed for estimating the odds ratios adjusted over strata, and Breslow–Day methods were used to examine odds ratio homogeneity across strata. Within strata, Fisher’s exact test (two-sided) was used. All reported odds ratios were adjusted across strata of ethnicity unless limited to a specified ethnic group. Data analyses were done using SAS 9.1.3.

Results

A total of 899 ovarian cancer cases and 931 controls were genotyped for the *MLH1* promoter nt-93 G>A polymorphism. The mean age of cases was 56.7 years at diagnosis (SD 9.9 years) and was 56.9 years for controls (SD 11.6 years) at blood sampling ($p = 0.8$, for difference). 22% of the cases had a positive family history for epithelial ovarian cancer. All cases were tested for BRCA1 and BRCA2 mutations; 7% had a BRCA1 mutation and 6% had a BRCA2 mutation (Table 1).

The *MLH1* nt-93 A allele was present in 43% of cases and 31% of controls (OR=1.5; 95% CI: 1.3–1.9; $p=0.00005$). This increased risk was present in all histologic subgroups except clear cell (Table 2). When stratified for age of diagnosis, the increased risk of ovarian cancer was statistically significant in women over 50 years of age (OR=1.5, 95% CI 1.2–2.0; $p=0.0006$). The mean age of diagnosis of ovarian cancer was 56.9 years for women with an A allele and 56.9 years for women with the GG genotype. In contrast, the age of diagnosis was 50.7 years for women with a BRCA1 mutation and 56.9 years for women with a BRCA2 mutation.

There appeared to be no modification of effect based on family history of cancer (Table 3). The association with increased risk was present in all ethnic groups (Table 3), although only reaching statistical significance in the largest (other white) group (OR = 1.5, 95% CI 1.2–1.8; $p = 0.0007$). The association was present in women without a BRCA mutation, but not in women with a BRCA mutation.

Discussion

We have identified an association between a single nucleotide polymorphism in the promoter region of the MMR gene *MLH1* (nt-93 G>A) and an increased risk of epithelial ovarian cancer (OR 1.5, $p = 0.0006$). Although the risk elevation is modest, the variant is common and is present in 31% of women in Canada. Based on the lifetime risk of ovarian cancer in US white women to be 1.5% to age 74 [1] and the observed odds ratio of 1.5, we estimate that carriers of this polymorphic variant have a 2.3% lifetime risk of epithelial ovarian cancer by 74 years.

Functional studies have shown that the promoter region of the *MLH1* gene, from nt-184 to the transcription start site, is essential for the transcription of the *MLH1* gene [13]. Thus the G>A transformation of the nt-93 *MLH1* promoter sequence may affect the transcriptional activation of the gene. Alternatively, this SNP may contribute to gene dysfunction by altering transcription through epigenetic changes. It has been suggested that this G>A nt-93 polymorphism may increase the susceptibility of the promoter sequence to methylation [14–16]. Studies of epithelial ovarian cancer display a variation in the frequency of *MLH1* promoter methylation from 9–20% [16–20]. The epigenetic modification occurs at the cytosines of the CpG dinucleotides, which often occur in clusters, called CpG islands. The *MLH1* nt-93 G>A polymorphism is located within a CpG island, adjacent to CpG sites that are able to undergo methylation [21].

In a small study by Willner et al. 20% of ovarian epithelial carcinomas displayed hypermethylation of the *MLH1* promoter [16]. Interestingly, endometrioid carcinomas

dominated the methylated group. However, promoter methylation was also identified in tumours of serous origin. In our study, the proportion of patients carrying the rare *MLH1* nt-93 A allele with serous, mucinous and endometrioid tumours was 42.5%, 45.9% and 42.9% respectively. It would be of interest to know whether or not there is an association between the presence of the *MLH1* nt-93 G>A allele and *MLH1* promoter methylation.

In summary, we have identified a polymorphism in the promoter region of *MLH1* which is associated with an increased risk of epithelial ovarian cancer in a mixed white North American population. Carriers of the rare *MLH1* nt-93 A allele had an increased risk of ovarian cancer (OR 1.5 $p=0.00005$). We estimate that carriers of this polymorphic variant have a 2.3% lifetime risk of epithelial ovarian cancer by 74 years. The role of this promoter polymorphism in malignancy is supported by other recent studies identifying an association with endometrial and colon cancers [11–14]. All three cancers are the principal components of the hereditary non-polyposis colon cancer syndrome.

Acknowledgments

We thank the National Cancer Institutes of Canada, the Canadian Institutes of Health Research and Genome Canada for supporting this project. Also supported in part by US National Cancer Institute Grants 5R01 CA063682 (to H.A.R.) and 5R01 CA063678 (to S.A.N.). We thank Isabel Fan and Linda Bradley for their contributions to the study, and Mehrdad Yazdanpanahand Maretta Chase for the technical support. Ian Harley is supported by the Department of Oncology, Queens University of Belfast, Northern Ireland.

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Table 1

Association between ovarian cancer risk and MLH1 nt-93, by histologic subtype

Histology	Genotype	Controls	Cases	OR (95%CI)	P-value
Other	GG	532	41	1	
	GA/AA	206/38	28/6	1.68 (1.04–2.73)	0.03
Clear cell	GG	532	33	1	
	GA/AA	206/38	15/3	1.00 (0.54–1.86)	1.00
Endometrioid	GG	532	101	1	
	GA/AA	206/38	59/17	1.55 (1.11–2.18)	0.01
Mucinous	GG	532	40	1	
	GA/AA	206/38	27/7	1.78 (1.09–2.91)	0.02
Papillary/serous	GG	532	268	1	
	GA/AA	206/38	168/29	1.54 (1.21–1.95)	0.0005
Overall	GG	532	483	1	
	GA/AA	206/38	297/62	1.54 (1.25–1.89)	0.00005
	No result	155	57		
	Total	931	899		

Table 2

Association between ovarian cancer risk and MLH1 nt-93, by age and family history.

Subgroup	Genotype	Controls	Cases	OR ^a (95%CI)	P-value
Age ≤ 50	GG	461	121	1	
	GA/AA	183/31	66/9	1.34 (0.96–1.86)	0.09
Age > 50	GG	461	283	1	
	GA/AA	183/31	173/28	1.53 (1.20–1.95)	0.0006
Family history negative	GG	461	316	1	
	GA/AA	183/31	188/28	1.47 (1.16–1.87)	0.001
Family history positive	GG	461	88	1	
	GA/AA	183/31	51/9	1.47 (1.02–2.12)	0.04
BRCA mutation	GG	461	48	1	
	GA/AA	183/31	25/6	1.39 (0.86–2.25)	0.18
No BRCA mutation	GG	461	322	1	
	GA/AA	183/31	196/29	1.51 (1.19–1.90)	0.0007

^aAll cases and controls in this table were from ethnic group "Other White".

Table 3

Association between ovarian cancer risk and MLH1 nt-93, by ethnic group

Ethnicity	Genotype	Controls	Cases	OR (95% CI)	P-value
Other white	GG	461	404	1	
	GA/AA	183/31	239/37	1.47 (1.18–1.84)	0.0007
French-Canadian	GG	41	43	1	
	GA/AA	14/1	23/7	1.91 (0.90–4.05)	0.10
Other/mix	GG	9	27	1	
	GA/AA	5/5	28/17	1.50 (0.54–4.16)	0.44
Unknown	GG	21	9	1	
	GA/AA	4/1	7/1	3.73 (0.96–14.6)	0.09
Overall	GG	532	483	1	
	GA/AA	206/38	297/62	1.54 (1.25–1.89)	0.00005
	No result	155	57		
	Total	931	899		