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Association of myeloperoxidase with ovarian cancer

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Abstract

Myeloperoxidase (MPO) is an oxidant generating enzyme normally restricted to myeloid cells, however aberrant MPO expression has been found to occur in non-myeloid cells in some disease states. The functional -463GA promoter polymorphism alters MPO expression levels. The -463G is within an SP1 binding site and is associated with higher gene expression. The G allele is most frequent with ~62% of European populations being GG homozygotes. The GA polymorphism has been associated with risk or survival in a variety of cancers including lung and breast cancer. In this study we determined the frequency of the -463G/A polymorphism in 230 ovarian cancer patients, 75 patients with borderline ovarian tumors, and 299 healthy controls. The GG genotype was found to be overrepresented in patients with early stage ovarian cancer (83.3% GG, p = 0.008) as compared to healthy controls (62% GG), suggesting MPO oxidants may increase risk. Immunohistochemical analysis revealed MPO expression in a subset of columnar ovarian epithelial carcinoma cells in early stage carcinomas.

Keywords

ovarian cancer; polymorphism; myeloperoxidase; oxidative stress; lymph node; FIGO

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Introduction

Ovarian cancer is the fourth most common cancer in women. While mutations in BRCA1 and BRCA2 are risk factors for familial ovarian cancer, the causal agents for sporadic ovarian cancers, representing the majority of cases, are relatively unknown [1–4]. Higher numbers of lifetime ovulations are one risk factor, reflecting the cell division required to repair the recurrent disruption of the ovarian epithelial layer. Reactive oxygen species generated by inflammatory cells at sites of ovulation have been suggested to lead to DNA mutations increasing cancer risk [5–7]. The process of ovulation is accompanied by the infiltration of neutrophils and monocyte-macrophages in the follicle wall, theca, and corpus luteum. These cells release inflammatory cytokines and oxidizing agents including myeloperoxidase (MPO). MPO is present at high levels in neutrophils and monocytes, and catalyzes reactions with hydrogen peroxide and chloride to produce hypochlorous acid (HOCl), a potent microbicidal agent that damages DNA, proteins, and lipids [8–12]. MPO also generates chloramines, reactive nitrating agents and free radicals leading to lipid peroxidation [13, 14]. MPO expression is normally restricted to myeloid precursors, however recent studies have found aberrant expression of MPO in mature macrophages in atherosclerosis [15] and some non-myeloid cells in neurodegenerative diseases [13, 15, 16]. This atypical expression can lead to oxidative damage to normal tissues contributing to atherosclerosis [13, 17, 18], Alzheimer's disease [16, 19], and some types of cancer [20]. Earlier studies have also found evidence implicating MPO in the initiation or progression of ovarian cancer [21–23].

MPO has been linked to risk or outcome for a variety of cancer types through association of the -463G/A promoter polymorphism [20, 24–31] This polymorphism (rs2333227) is situated 463 bp upstream of the transcription iniitation site within a primate-specific Alu element that encodes several sites recognized by SP1 and members of the nuclear receptor superfamily of transcription factors including estrogen receptor (ER), retinoic acid receptor (RAR), and peroxisome proliferator activated receptor (PPARα/γ) [32–34]. The -463G site enhances binding by Sp1 while -463A enhances binding by estrogen receptor [33–35]. The G allele is several fold higher expressing than the A allele [33, 34, 36] and the GG genotype is most frequent at 48-65% of European or American populations [19, 20, 29]. The higher expressing GG genotype has been associated with increased risk for a number of types of cancer [20, 37]. A recent meta-analysis of breast cancer studies, including 2975 cases and 3427 controls, associated the AA genotype with significantly decreased risk for breast cancer in pre-menopausal women (OR 0.56, p=0.03)[25], thereby associating the higher expressing GG genotype with increased risk. Conversely, in two studies, the GG genotype enhanced survival in early stage breast cancer patients undergoing chemotherapy. Patients with the G allele (GG or GA) had more than a two-fold reduction in risk of recurrence or death [27, 28]. These studies suggest that MPO can both promote breast cancer through DNA damage while enhancing the tumoricidal activities of chemotheraputic drugs.

In a study of ovarian cancer patients, the lower expressing A allele (GA/AA genotypes) was previously found to be associated with a small reduction in risk (OR=0.72) although this trend did not reach statistical significance [22]. Other studies have found higher levels of MPO in gynaecological or ovarian cancer tissue [21, 23, 38] as well as evidence that MPO

promotes nitrosylation of caspase-3, potentially inhibiting apoptosis and enhancing survival of ovarian cancer cells [21].

These studies raise the possibility that MPO generated oxidants affect incidence or progression of ovarian cancer. In this study we investigated the expression of MPO in ovarian cancer tissue and the association of the -463GA polymorphism with cancer incidence. The findings show that MPO is robustly expressed in a subset of ovarian carcinoma cells, and that the higher expressing GG genotype is overrepresented in early stage (FIGO I) cancer.

Materials and Methods

Samples and patients

EDTA-blood samples from 230 sporadic ovarian carcinoma patients and 75 patients with ovarian borderline tumors were collected at the Department of Obstetrics and Gynaecology, Medical University of Vienna, Austria; Department of Obstetrics and Gynaecology, Charité, Berlin, Germany; and University of Medical Sciences, Poznan, Poland during 1991 to 2004. EDTA-blood samples from healthy women or women without any indication of ovarian malignancies were collected from the Department of Obstetrics and Gynaecology, Department of Blood Group Serology and Transfusion Medicine, Medical University of Vienna, Austria and Department of Obstetrics and Gynaecology, University of Ulm Medical School, Ulm, Germany. The characteristics of the carcinomas, borderline tumors, and the age of patients at diagnosis are shown in tables 1 and 2. Histopathological diagnosis and clinical staging were classified according to the criteria of the International Federation of Gynaecology and Obstetrics (FIGO) by pathologists in the corresponding institutions. Briefly, FIGO stage 1 is limited to one or both ovaries; stage II is limited to the uterus and pelvic structures; stage III includes extension to the small bowel or omentum; stage IV includes distant metastases to the liver or outside the peritoneal cavity. Only epithelial ovarian carcinomas of serous, mucinous, or endometrioid histological type were involved in this study. Patients underwent hysterectomy, bilateral salpingo-oophorectomy, pelvic and/or paraaortic lymphadenectomy, appendectomy, or omentectomy. All patients with FIGO stage Ic to IV received a platinum-based chemotherapy. All patients were followed-up with vaginal and rectal palpation, serum marker evaluation, and vaginal cytology in a three months interval and abdominal CT scan in a twelve months interval. Whenever possible, recurrence was proven histologically or otherwise indicated by X-ray, computer tomography and/or tumor markers as measurable disease. The median observation time was 67 months. All procedures were approved by the responsible institutional ethical committees.

DNA preparation and genotyping of MPO polymorphism

The G/A polymorphism at -463 of the MPO gene (rs2333227) was determined by digestion of a PCR amplified DNA fragment with Aci1 restriction enzyme that cuts at the G site [19, 26]. A confirmatory method was pyrosequencing using a Pyrosequencer PSQ 96 and the PSQ 96 SNP Reagent Kit (Uppsala, Sweden). DNA was isolated from blood using commercial kits (DNA Extraction System I; ViennaLab, Vienna, Austria). The primers MPO-SE 5’-ATCTTGGGCTGGTAGTGC-3’ and MPO-AS 5’-

CCACATCATCAATTATTTCC-3' were used to amplify a 238bp fragment of the *MPO* gene (GenBank accession no. M19507). MPO-SE was biotinylated. PCR was carried out in a total volume of 25µl including 25ng template, 5pmol of each sense and antisense primers and puReTaq Ready-To-Go PCR Beads (Amersham Biosciences UK Limited, UK), which contain 2.5 units of puReTaq DNA polymerase, 10mM Tris-HCl (pH 9.0 at room temperature), 50mM KCl, 1.5mM $MgCl_2$, 200µM dATP, dCTP, dGTP and dTTP, and stabilizers, including BSA. PCR was performed on a Perkin-Elmer GeneAmp PCR system 9600 with 40 cycles at 94°C for 30 seconds, at 51°C for 30 seconds and 72°C for 30 seconds. The reaction was preceded by a primary denaturation step at 94°C for 1 minute and incubated at 72°C for 7min at last.

25µl PCR product was used for pyrosequencing according to the instruction of the manufacturer. 5 pmol of the sequencing primer MPO-SEQ 5'-CCTCAAGTGATCCACC -3' was applied to detect the polymorphism.

Statistical analysis

Association of genotypes with histological type, differentiation grade, FIGO stage and nodal status of the ovarian malignancies and age of the patients, which was dichotomized at 50 years, was assessed using the Chi-square test. Genotype distributions and allele frequencies were compared between patients with ovarian malignancies and healthy women using the Chi-square test. In all analyses, the Chi-square test was replaced by Fisher's exact test whenever an expected cell frequency was lower than 5. In either subpopulation, violations of the Hardy-Weinberg-assumptions were statistically tested by comparing the observed genotype distribution with that expected under the Hardy-Weinberg equilibrium, using an exact permutation test. 95% confidence intervals for allele frequencies are based on 10,000 bootstrap resamples of individuals. The association of *MPO* genotypes with the risk of nodal involvement was expressed as odds ratio (OR), estimated by exact conditional logistic regression. These ORs were computed for the additive (allele-dose) model, and the dominance (carrier vs. non-carrier) models. 95% confidence intervals (CI) for the OR were computed using the mid-P method. Disease-free survival is defined as time between diagnosis of disease and recurrence or distant metastasis. Overall survival is defined as time from diagnosis of disease to death of a patient. The association of *MPO* genotype with disease-free survival and overall survival was assessed by estimating survival curves through the method of Kaplan-Meier [20], which was compared by the log-rank test. The Cox regression model [21] was used to estimate crude hazard ratios and hazard ratios adjusted by FIGO stage, nodal status, age, and differentiation grade. The statistical software package SAS V9.1 (2003 SAS Institute Inc., Cary, NC) was used. A p value of <0.05 was considered statistically significant.

Immunohistochemistry

Paraffin sections of human ovarian cancer tissue were cleared by xylene and ethanol prior to heat induced antigen retrieval in 10 mM sodium citrate buffer, 0.05% Tween 20, pH 6.0. Sections were incubated in 10% normal goat serum for one hour, followed by incubation for 12 hours in primary antibodies in phosphate buffered saline with 0.05% Tween 20 (PBST) and 10% normal goat serum. Primary antibodies were rabbit anti-human myeloperoxidase

(DAKO, 1:1000) or mouse anti-human CD68 (DAKO, clone EBM11, 1:1000). Following incubation, the sections were washed in PBST for 2 hours prior to incubation with secondary fluorescent antibodies including Alexa Fluor 488 conjugated (green) goat anti-rabbit IgG or Alexa Fluor 594 (red) goat anti-mouse IgG, both at 1:3000 dilution. After washing, confocal images were obtained with a DeltaVision Deconvolution microscope with multiple fluorochrome (488, 594, DAPI, Cy-5, YFP) and Z series capabilities. Appropriate controls included staining of adjacent slides with secondary antibodies alone (no primary antibody) and staining with irrelevant primary antibodies (Invitrogen isotype controls for mouse or rabbit IgG).

Nonfluorescent immunostaining of paraffin sections was carried out with DAKO rabbit anti-MPO (1:1000) in PBST with 10% normal goat serum, followed by biotinylated goat anti-rabbit IgG (Vector) (1:200, 1 hour) and avidin-biotin conjugates (Vector Elite ABC system) (1:200, 2 hr), and developed with Vector SG peroxidase substrate (brown). Images were obtained with an Olympus BX 21 microscope with 20x, 40x, and 100x oil immersion lenses.

Results

Analysis of myeloperoxidase expression in ovarian cancer cells

Immunohistochemical analysis of ovarian cancer tissue revealed robust levels of MPO expression in some of the ovarian cancer cells. Confocal images of FIGO stage 1 ovarian carcinoma detected MPO in columnar epithelial carcinoma cells (Fig.1A) that were clearly distinct from nearby CD68-positive monocyte-macrophages which lacked MPO. In the same tumor, MPO was similarly detected in epithelial cancer cells in the absence of adjacent CD68-positive macrophage-monocytes (Fig. 1B). Detection of MPO in ovarian cancer cells was unexpected because MPO protein is normally restricted to myeloid cells, including neutrophils, monocytes and some reactive macrophages [10, 15]. In other regions of this tumor, MPO colocalized with CD68 in macrophages infiltrating the epithelial layers (Fig. 1C), indicating MPO expression can be induced in some macrophages closely associated with ovarian cancer cells. Appropriate controls showed no staining in the absence of primary antibodies or presence or irrelevant primary antibodies (Fig.1. D-F). In borderline tumors, MPO was similarly expressed in a subset of epithelial cells in papillary formations, and was found to be strongly expressed in papillary regions undergoing loss of epithelial organization (Fig. 1. G,H). In advanced serous carcinomas (FIGO stage III), MPO was present at high levels in regions containing ovarian cancer cells as well as infiltrating neutrophils and monocytes (Fig. 1I). Overall, these findings clearly show that MPO can be expressed in subsets of ovarian cancer cells in early to later stage tumors, raising the possibility that MPO oxidants could impact tumor cell growth and survival.

Determination of association of the -463GA MPO promoter polymorphism with ovarian cancer cases and controls

One means to gain information as to the effects of MPO expression levels on ovarian cancer is to determine the frequencies of the -463GA promoter polymorphism. This is a functional polymorphism that has been demonstrated to alter gene expression levels and to be associated with risk for a number of cancers as well as atherosclerosis and

neurodegenerative diseases [13, 15, 19, 35, 39]. Here we compared the MPO genotype frequencies in patients with early to late clinical stages of ovarian cancer as defined by FIGO stages I to IV (Table 1) as well as borderline cases (Table 2). The MPO genotype distribution was found to be significantly different with regard to FIGO stage. The percentage of GG genotype was higher in patients with early stage (FIGO I) as compared to patients with later stages (FIGO II to IV)(p=0.014) (Table 1). Consistent with that finding, the GG genotype was more frequent in patients lacking lymph node involvement (72.2%) than in patients with lymph node involvement (53.7%)(p=0.012)(Table 3). The genotype distribution of FIGO I cases was also significantly different from borderline tumors (GG 57.3%) (p=0.01) or healthy controls (GG 62.5%)(Table 4) (p=0.024). Analysis of allelic frequencies showed the A-allele frequency for FIGO I stage carcinomas was 8%, contrasting significantly with 22% A allele in advanced FIGO stage II-IV cases (p=0.004), 23% in borderline tumors (p=0.004), and 26% in healthy controls (p=0.007) (Tables 1 and 4). There was no association of MPO genotype with histological type of cancer (serous, mucinous, or endometrioid), or age at diagnosis (Table 1).

Discussion

These findings show that the higher expressing -463GG genotype is more frequent in FIGO I early stage carcinoma suggesting the G allele increases risk for ovarian cancer. These findings are consistent with an earlier study in which the lower expressing A allele was associated with reduced risk for ovarian cancer [22], although that finding did not reach statistical significance. Together these studies suggest the atypical expression of this normally myeloid specific MPO gene in ovarian epithelial cells could lead to oxidation damage and mutations that increase risk of cancer.

The higher expressing GG genotype was not overrepresented in later stage II-IV cancers. Current models of ovarian cancer propose that early grade (Type I) and high grade (Type II) ovarian carcinomas are distinct diseases, arising by different genetic mutations [2, 40]. Type I carcinomas are more often associated with KRAS and BRAF mutations, while Type II carcinomas tend to have mutations in p53 and BRCA1/2 genes [2]. Type I carcinomas appear to develop in incremental steps from borderline adenomas, while Type II cancers appear to arise from the epithelium as a rapidly growing cancer without a precursor state. If Type I and II are distinct diseases, this could explain why GG genotype is associated with early but not later stages. A second possible explanation is that higher MPO expression in early stage GG carcinomas results in oxidative damage that impairs cell survival, such that fewer GG cancer cells survive to advanced stages. As a third possibility, higher levels of MPO in invading GG neutrophils and monocyte-macrophages could promote the killing of early stage cancer cells, reducing the number of GG cases that advance to stages II-IV.

The finding of MPO in a subset of clearly defined epithelial carcinoma cells was unexpected. A prior study detected MPO in ovarian cancer tissue in low magnification images of low resolution in which the localization of MPO in individual cells was unclear [21]. Our confocal studies shown here reveal discrete expression in subsets of epithelial cancer cells rather than uniform overall expression. MPO is detected in early carcinoma cells as well as in distinct focal areas in borderline and advanced stage cancers. These

findings are consistent with prior studies showing that the human MPO gene can be aberrantly expressed in subsets of non-myeloid cells in disease states such as Alzheimer's and Parkinson's disease [13, 16, 19, 41].

The association of the higher expressing MPO GG genotype with greater risk for early stage ovarian cancer is consistent with DNA damage. MPO is known to create oxidants that damage DNA. The major MPO product, HOCl, results in oxidation of pyrimidine bases and chlorination of cytosine in bronchial epithelial cells [42]. MPO is involved in the bioactivation of inhaled polycyclic aromatic hydrocarbons to generate DNA binding metabolites that are mutagenic [43, 44]. HOCl inhibits repair of DNA strand breaks and nucleotide excision [45–47]. The detection of higher levels of MPO in cancer tissue is consistent with a role in carcinogenesis. Patients with colorectal tumors were found to have a higher number of MPO-positive cells in normal mucosa than did controls [48, 49]. Ovarian cancer tissue was found to have higher MPO levels than benign growths or inflammatory tissue [23]. Serum MPO levels were also found to be higher in stage II-IV ovarian cancer cases [23]. Consistent with the association of higher levels of MPO with cancer risk, the higher expressing MPO G allele has been linked to increased risk for a number of cancers including lung [24, 31, 50, 51], breast [28, 52], ovarian [22], bladder [53], larynx [29], as well as myeloid leukemia [26].

In summary, these studies provide evidence that MPO is atypically expressed in a subset of ovarian cancer cells. A second finding is that the higher expressing MPO GG genotype is associated with early stage carcinoma suggesting MPO oxidants increase cancer risk, consistent with an earlier study associating the low expressing A-allele with reduced risk [22]. These findings are consistent with a model in which MPO oxidants damage DNA thereby increasing risk of mutations promoting cancer. Further studies are needed to determine the role of MPO expression in ovarian cancer.

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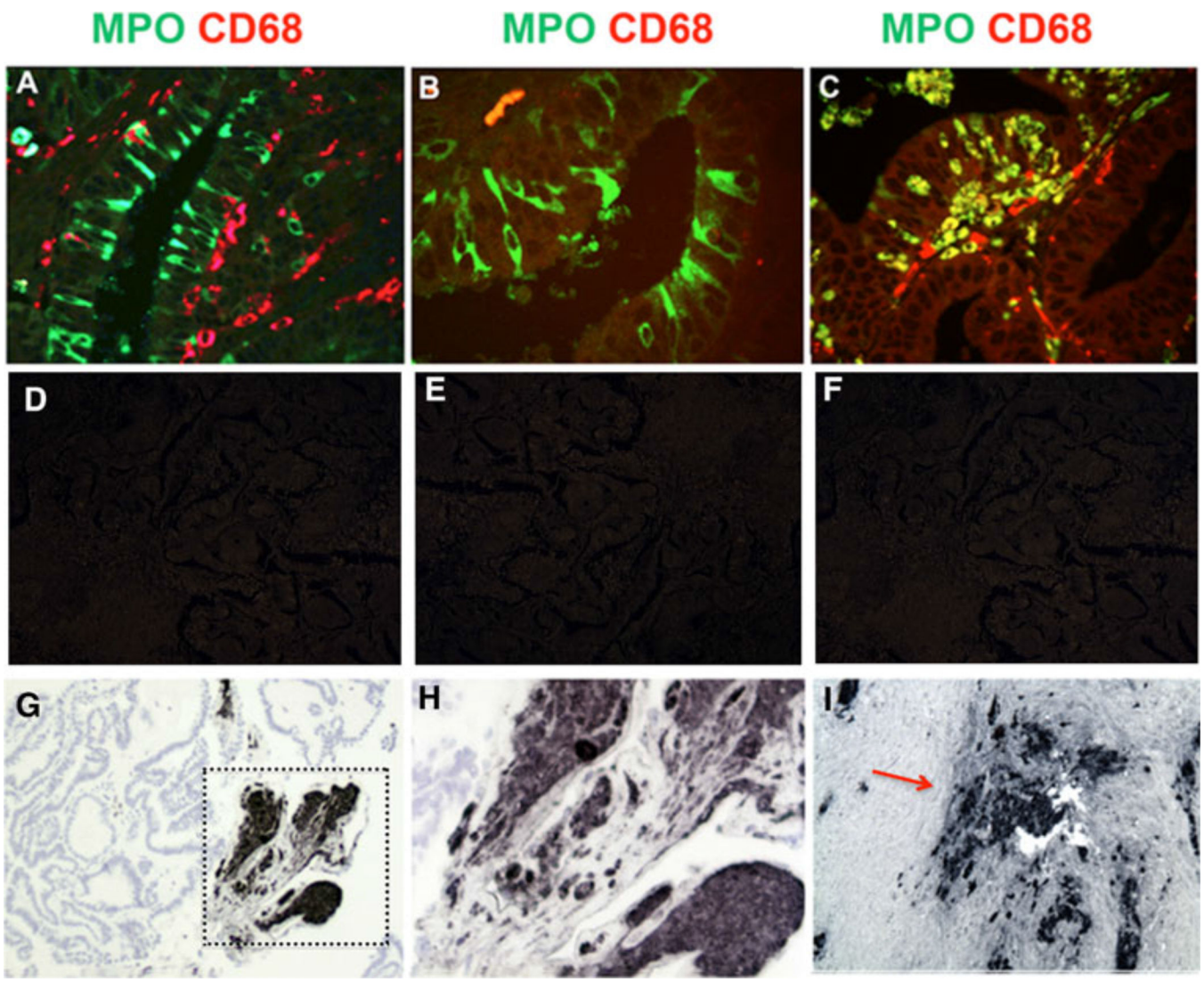

Figure 1.

MPO expression in epithelial ovarian cancer cells.

A. Digital confocal image (Delta Vision microscope) of stage 1 ovarian endometriod cancer shows MPO (Alexa Fluor 488, green) immunostaining in columnar ovarian epithelial cancer cells, and nearby CD68 positive (Alexa Fluor 594, red) monocyte-macrophages lacking MPO co-staining.

B. Digital confocal image of another region of the same tumor showing MPO (Alexa Fluor 488, green) positive epithelial cancer cells in the absence of nearby CD68 positive macrophages (Alexa Fluor 594, red).

C. Digital confocal image from the same tumor showing colocalization (yellow) of MPO (Alexa Fluor 488, green) and CD68 (red) in macrophages invading an epithelial structure.

D. Control image of a nearby section of the same ovarian cancer section seen in panel A showing lack of fluorescent staining with the Alexa Fluor 488 anti-rabbit IgG in the absence of the primary antibody.

E. Control image of a nearby section stained with an irrelevant rabbit IgG antibody and the Alexa Fluor 488 anti-rabbit IgG.

F. Control image of a section near that seen in panel C stained with an irrelevant mouse IgG control antibody and Alexa Fluor 594 anti-mouse IgG.

G. MPO immunoperoxidase staining in a papillary region of a borderline tumor.

H. Boxed area in panel D is shown at higher magnification showing MPO immunostaining in a complex cellular structure.

I. High levels of MPO immunostaining (red arrow) in a stage 3 serous tumor in both tumor cells and infiltrating neutrophils and monocyte-macrophages.

Table 1

Characteristics of 230 sporadic ovarian carcinomas and patients

	Number of samples	GG (%)	GA (%)	AA (%)	p value
Histological type					
Serous carcinoma	192	124 (64.6)	61 (31.8)	7 (3.7)	
Mucinous carcinoma	12	6 (50.0)	6 (50.0)	0 (0.0)	0.619
Endometrioid carcinoma	26	19 (73.1)	7 (26.9)	0 (0.0)	
FIGO					
I	42	35 (83.3)	7 (16.7)	0 (0.0)	**0.014**
II+III+IV	180	108 (60.0)	65 (36.1)	7 (3.9)	
Unknown	8	6 (75.0)	2 (25.0)		
N (Nodal status)					
Negative	115	83 (72.2)	30 (26.1)	2 (1.7)	**0.025**
Positive	67	36 (53.7)	28 (41.8)	3 (4.5)	
Unknown	48	30 (62.5)	16 (33.3)	2 (4.2)	
Age					
≤50 years	76	55 (72.4)	18 (23.7)	3 (4.0)	0.137
>50 years	154	94 (61.0)	56 (36.7)	4 (2.6)	
Total	**230**	**149 (64.8)**	**74 (32.2)**	**7 (3.0)**	

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Table 2

Characteristics of 75 borderline ovarian tumors and patients

	Number of samples	GG (%)	GA+AA (%)	p value
Histological type				
Serous carcinoma	56	30 (53.6)	26 (46.4)	0.329
Mucinous carcinoma	18	12 (66.7)	6 (33.3)	
Endometrioid carcinoma	1	1 (100.0)		
Age				
≤ 50 years	33	17 (51.5)	16 (48.5)	0.367
>50 years	42	26 (61.9)	16 (38.1)	
Total	**75**	**43**	**32**	

Table 3

Risk of lymph node involvement

	Number of lymph node negative patient (%)	Number of lymph node positive patient (%)	Odd ratio (95% CI)
GG	83 (72.2)	36 (53.7)	
GA	30 (26.1)	28 (41.8)	[1]2.06 (1.17-3.63), **p=0.012**
AA	2 (1.7)	3 (4.5)	
Total	115 (100.0)	67 (100.0)	[2]2.22 (1.18-4.20), **p=0.013**

[1] Additive model, analyzing A-allele effect (AA vs. GA vs. GG);

[2] Dominance model, comparing carriers of A-allele with non-carriers (AA+GA vs. GG)

Table 4

Genotype distribution and allele frequencies of the -463 polymorphism in the *MPO* gene.

	Total	GG (%)	GA (%)	AA (%)	GA+AA (%)	A allele frequency (95% CI)	HW*
Control	299	187 (62.5)	100 (33.4)	12 (4.0)	112 (37.5)	0.21 (0.18-0.24)	0.86
Borderline Tumor	75	43 (57.3)	29 (38.7)	3 (4.0)	32 (42.7)	0.23 (0.17-0.30)	0.74
Ovarian Cancer	230	149 (64.8)	74 (32.2)	7 (3.0)	81 (35.2)	0.19 (0.16-0.23)	0.68

*HW: exact p-value for testing Hardy-Weinberg equilibrium