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Research Article

Molecular Signatures of Epithelial Ovarian Cancer: Analysis of Associations with Tumor Characteristics and Epidemiologic Risk Factors

Joellen M. Schildkraut[1,6], Edwin S. Iversen[2,6], Lucy Akushevich[6], Regina Whitaker[3], Rex C. Bentley[4], Andrew Berchuck[3,6,7], and Jeffrey R. Marks[5,6,7]

Abstract

Background: Six gene expression subtypes of invasive epithelial ovarian cancer were recently defined using microarrays by Tothill and colleagues. The Cancer Genome Atlas (TCGA) project subsequently replicated these subtypes and identified a signature predictive of survival in high-grade serous (HGS) cancers. We previously validated these signatures for use in formalin-fixed paraffin-embedded tissues. The aim of the present study was to determine whether these signatures are associated with specific ovarian cancer risk factors, which would add to the evidence that they reflect the heterogeneous etiology of this disease.

Methods: We modeled signature-specific tumor characteristics and epidemiologic risk factor relationships using multiple regression and multivariate response multiple regression models in 193 patients from a case–control study of epithelial ovarian cancer.

Results: We observed associations between the Tothill gene expression subtype signatures and both age at diagnosis ($P = 0.0008$) and race ($P = 0.008$). Although most established epidemiologic risk factors were not associated with molecular signatures, there was an association between breast feeding ($P = 0.024$) and first-degree family history of breast or ovarian cancer ($P = 0.034$) among the 106 HGS cases. Some of the above associations were validated using gene expression microarray data from the TCGA project. Weak associations were seen with age at menarche and duration of oral contraceptive use and the TCGA survival signature.

Conclusions: These data support the potential for genomic characterization to elucidate the etiologic heterogeneity of epithelial ovarian cancer.

Impact: This study suggests that molecular signatures may augment the ability to define etiologic subtypes of epithelial ovarian cancer. *Cancer Epidemiol Biomarkers Prev; 22(10); 1709–21. ©2013 AACR.*

Introduction

Ovarian cancer is a heterogeneous disease as shown through associations with family history of cancer, genetic risk, and the histopathology of this disease. In addition, ovarian cancer is more common among Whites and more lethal among Blacks. The most common histology is epithelial ovarian cancer comprising 95% of invasive cancers. Among the invasive epithelial ovarian cancers, there are several subtypes of which the serous subtype is the most common followed by endometrioid, clear cell, and mucinous cancers. Some risk factor associations are specific to histologic subtypes of this disease such as endometriosis and endometrioid/clear cell cancers (1) and smoking and mucinous cancers (2).

Tothill and colleagues (3) recently reported evidence for six distinct molecular subtypes of epithelial ovarian cancer based on microarray gene expression profiling of 285 serous and endometrioid tumors of the ovary, peritoneum, and fallopian tube. Four of these expression subtypes corresponded to most of the high-grade serous (HGS) cancers, whereas the other two expression subtypes were enriched for low malignant potential and low-grade endometrioid subtypes. More recently, The Cancer Genome Atlas (TCGA) project identified four-gene expression "intrinsic subtypes" of HGS ovarian cancer based on patterns of gene expression (mesenchymal, immunoreactive, differentiated, and proliferative) that are highly congruent with the subtypes described by Tothill and colleagues (4). In addition, a signature predictive of survival was identified within the group of HGS cancers. To

Authors' Affiliations: Departments of [1]Community and Family Medicine, [2]Statistical Science, [3]Obstetrics and Gynecology, Division of Gynecologic Oncology, [4]Pathology, and [5]Surgery, [6]Duke Cancer Institute, and [7]Institute for Genome Sciences and Policy, Duke University Medical Center, Durham, North Carolina

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Corresponding Author: Joellen M. Schildkraut, Department of Community and Family Medicine, Duke University Medical Center, Box 2715, Durham, NC 27710. Phone: 919-681-4761; Fax: 919-681-4766; E-mail: schil001@mc.duke.edu

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date, there have been no reports examining the associations between molecular subtypes and patient demographic characteristics or epidemiologic risks factors.

We sought to determine whether the six molecular subtypes of epithelial ovarian cancer identified by Tothill and colleagues (3) and the TCGA-derived survival signature (4) are related to risk factors or demographic variables in the population-based North Carolina Ovarian Cancer Study (NCOCS).

Materials and Methods

Study subjects

Study subjects are a subset of invasive epithelial ovarian cancer cases selected from participants in the NCOCS, a population-based case–control study conducted in a 48-county region of North Carolina. A detailed description of the study has been published previously (5, 6). Briefly, newly diagnosed cases were identified through the North Carolina Central Cancer Registry using rapid case ascertainment, and physician's approval was obtained before the patients were contacted. Eligible cases, ages 20 to 74 years, were diagnosed with epithelial ovarian cancer between 1999 and 2007. All cases underwent standardized histopathologic review by the study pathologist (R.C. Bentley) to confirm diagnosis. The response rate among eligible cases was 65%. Although the response rate was low, the distribution of histologic subtypes and stage at diagnosis did not seem to be significantly different among the responders compared with the nonresponders.

Subjects represent a subset of those with invasive epithelial ovarian cancer enrolled in the NCOCS and were selected because they were diagnosed with invasive epithelial ovarian cancer and because of availability of fresh-frozen tumor tissue and matched formalin-fixed paraffin-embedded (FFPE) tissue samples. Because of this requirement, all subjects in the current study were diagnosed at Duke University Medical Center (Durham, NC). There were 193 invasive epithelial ovarian cancer cases of which 106 were advanced-stage HGS ovarian cancers. This subset of subjects were found to have comparable distributions of age at diagnosis and epidemiologic risk factors compared with the remaining invasive ovarian cancer cases in the NCOCS. However, differences in the distributions of some characteristics were found in this subset, including lower proportions of African Americans and poorly differentiated cancers, and higher proportions of invasive serous and advanced-stage cancer.

The protocol was approved by the Duke University Medical Center Institutional Review Board and the human subjects committees at the Central Cancer Registry and each hospital where cases were identified.

Data and biospecimen collection

Nurse-interviewers obtained written-informed consent, administered a standardized questionnaire to all NCOCS participants, and drew a blood sample. A FFPE tissue sample was obtained for 95% of cases enrolled in the NCOCS. During the in-person interview, anthropometric measurements including height, weight, and waist and hip circumferences were also obtained. The questionnaire included information on established and suspected ovarian cancer risk factors including family history of cancer, menstrual characteristics, reproductive history, infertility, hormone use, and lifestyle characteristics such as smoking, alcohol consumption, and physical activity. A more detailed description of the data collection process was published previously (6).

Body mass index (BMI) was calculated as weight 1 year before diagnosis in kg/(height in meters)^2 and was categorized as <25, 25–<30, 30–<35, and $\geq$35 kg/m^2. Other variables included in the analysis were age at diagnosis (20–49, 50–59, 60–69, and 70–74 years), race (African American vs. non-African American), menopausal status (pre- or postmenopausal), age at last pregnancy (never pregnant, <24, 24–29, 30–34, or $\geq$35 years), number of live births (none, 1–2, 3–4, or $\geq$5), age at menarche (<12 and $\geq$12 years), a diagnosis of endometriosis (yes or no), years of oral contraceptive (never, <1, 1–<5, or $\geq$5), months breastfed (never, <6, 6–12, or >12), family history of breast or ovarian cancer in a first-degree relative (yes or no), and tubal ligation (yes or no). Characteristics of the tumor that were included in the analysis were diagnosis of primary site (ovary or peritoneum), tumor histologic subtype (serous, endometrioid, mucinous, clear cell, or other), tumor grade (well differentiated, moderately differentiated, and poorly differentiated), and stage at diagnosis (I/II or III/IV).

Gene expression analysis

All aspects of the generation of the gene expression data from these specimens are detailed in Sfakianos et al. (7). This publication describes a series of quality control steps for RNA extraction and derivation of gene expression using the whole-genome Illumina cDNA-mediated annealing, selection, extension, and ligation (DASL) platform (HumanRef-8 Bead Chip). For a subset ($n = 43$) of these samples, RNA from fresh-frozen tissue and paired archival FFPE derived RNA were compared (Affymetrix U133A for the frozen tissue vs. DASL for the FFPE). Briefly, the Affymetrix and Illumina data were preprocessed using the robust multi-chip average (RMA) (8) and limma (9) R/Bioconductor packages, respectively. DASL data for 13 of 43 paired samples and 4 of the NCOCS samples failed laboratory quality control and were removed from the analysis before preprocessing, leaving 30 paired samples with which to evaluate cross-platform concordance and 193 NCOCS samples for the association study. Affymetrix and Illumina probes were mapped to one another by RefSeq ID and the data were collapsed to their median in cases of many-to-one-matches. The resulting sets of Affymetrix- and Illumina-derived expression data were standardized separately so that each probe in each set had mean 0 and SD 1.

Construction of the subtype signatures is described in Sfakianos and colleagues (7). Briefly, we defined the signature of a cluster as a weighted sum of the normalized

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expression values for the overexpressed probes minus a weighted sum of the normalized expression values for the underexpressed probes published by Tothill and colleagues (4). To downweight poor performing DASL probes, we weighted each expression value in the signature by the square of its correlation between Affymetrix and DASL log expression estimates calculated using a concurrently analyzed external set of paired fresh frozen/FFPE samples after truncating negative correlations at zero. Hence, the larger the value of a tumor's cluster signature, the more likely it is to be of that subtype. We defined the TCGA survival signature as the weighted sum of normalized gene-level DASL expression values with weights equal to the product of the associated gene's published log hazard estimate (Supplementary Table S1; ref. 4) and its squared Affymetrix/DASL correlation in the external paired samples. We normalized all signatures to have mean 0 and SD 1.

Statistical analyses

We used multivariate response multiple regression models with unstructured error covariance matrices to simultaneously assess the relationship between ovarian cancer risk factors, tumor characteristics, and the six molecular subtypes of ovarian cancer. The six signatures, normalized to have mean 0 and SD 1 across samples, served as the response variables in these models. Hence, regression coefficients are interpreted as the expected difference in the signature, measured in SDs, associated with a unit change in the covariate. We examined the associations with tumor characteristics collectively while controlling for age at diagnosis. We conducted these analyses for all ovarian cancer cases in the study and in the subset of advanced-stage (stage III/IV) HGS (moderately to poorly differentiated) invasive cases. We also conducted analyses for each ovarian cancer risk factor individually while controlling for age for all histologic subtypes combined and restricting the analysis to advanced-stage HGS cancer.

To this end, we used a likelihood ratio test to assess evidence in favor of association between each risk factor and tumor characteristic variable of interest and the ensemble of subtype signatures. In particular, we tested H_0: the signatures are conditionally independent of the variable given age versus H_1: the signatures are conditionally dependent on the variable given age. The coefficients of the variable in the H_1 model were signature-specific as were all remaining parameters, including the intercepts, in the H_0 and H_1 models.

In addition, we used multivariable linear regression to model relationships between the TCGA survival signature and tumor characteristics and epidemiologic risk factors. We similarly conducted multivariable linear regression analyses to examine signature-specific associations between tumor characteristics and epidemiologic factors for each of the six subtype signatures defined by Tothill and colleagues (3).

Results

Tothill and colleagues (3) used unsupervised hierarchical clustering to assign epithelial ovarian cancers into six subtypes (C1–C6). These subtypes show enrichment for certain molecular and histologic features and were labeled accordingly: C1 are tumors with a high stromal content, C2 is defined primarily by an immune signature associated with tumor inflammatory response, the C3 signature is characterized as well differentiated and contains all of the low malignant potential cancers in the study but also some invasive serous cancers, C4 is an expression cluster that is characterized by the absence of other strong signatures and is labeled as cancers with a low stromal response, the C5 cluster is called mesenchymal or poorly differentiated and these are all HGS cancers, whereas C6 are low-grade cancers with endometrioid features. These categories are congruent with the four TCGA expression subtypes that were defined on HGS cancers only. The TCGA subtypes include differentiated, which overlaps with C3 (LMP) and C4 (low stroma), mesenchymal, which is analogous to the Tothill C1 high stromal signature, immunoreactive, which contains most of the inflammatory signature associated with C2, and proliferative characterized by genes associated with cell division some of which can be

Table 1. Pearson correlation coefficients and (*P* values) for molecular clusters (C1–C6) and the TCGA survival signature in invasive ovarian cancers ($N = 193$)[a]

	C1	C2	C3	C4	C5	C6	TCGA survival signature
C1	1.00	−0.024 (0.741)	−0.164 (0.023)	−0.931 (<0.0001)	−0.461 (<0.0001)	−0.423 (<0.0001)	−0.012 (0.873)
C2		1.00	−0.482 (<0.0001)	0.221 (0.002)	−0.687 (<0.0001)	−0.567 (<0.0001)	−0.217 (0.002)
C3			1.00	−0.039 (0.590)	0.086 (0.233)	0.488 (<0.0001)	−0.0202 (0.005)
C4				1.00	0.320 (<0.0001)	0.205 (<0.004)	0.013 (0.862)
C5					1.00	0.616 (<0.0001)	0.424 (<0.0001)
C6						1.00	0.158 (0.028)

[a]C1: high stromal response, C2: immunoreactive, C3: well differentiated, C4: low stromal response, C5: mesenchymal/undifferentiated, C6: endometrioid.

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found in Tothill C5 but also present in other HGS categories (C1, C2, and C4; ref. 4). Conversely, the two low-grade Tothill categories, C3 and C6, are characterized by a low proliferation signature.

For the current study, we derived quantitative signatures for each tumor belonging to a specific Tothill cluster (C1–C6), hence class membership is not a categorical yes or no. To determine whether the class signatures represent nonoverlapping biologic properties, we determined correlations between classes. If all cancers show unique class characteristics, then strong negative correlations would be expected. Although the stronger correlations between the molecular clusters we observe tend to be negative (see Tables 1 and 2), we do estimate some positive correlations, notably between the C6 signature and the C3, C4, and C5 signatures, indicating that not all signatures are mutually exclusive. This is consistent with the recent analysis of the TCGA data and indicates that membership in an expression subtype is not rigid and cancers can have characteristics of multiple classes (10). All subsequent analyses use this approach where each cancer is assigned a value for each cluster based on the combination of gene expression values that define that cluster.

The distributions of patient and tumor characteristics for invasive cases ($n = 193$) and for the subset of advanced-stage HGS tumors ($n = 106$) are found in Table 3. Most of the tumors were serous ovarian cancer (65.3%), stage III/IV at diagnosis (80.8%), and moderately to poorly differentiated at diagnosis (84.9%). Only 29 women (15%) in our sample reported their race as African American, with the majority of subjects reporting their race as Caucasian (81.8%). Eighty percent of the ovarian cancer cases were 50 years of age or older at diagnosis. Of the 193 cases in the study, 18.1% were nulliparous, 40.4% never used oral contraceptives, and 23.8% of the study subjects reported a family history of breast or ovarian cancer in a first-degree relative.

In the subset of women diagnosed with advanced-stage HGS tumors, compared with the combined histologic subtypes, a lower proportion had an early age at menarche less (<12 years), 19% versus 23.4% and a higher proportion have a primary site of the peritoneum, 15% versus 10%, respectively.

Tumor characteristics

The mixed model testing for heterogeneity includes age at diagnosis, histologic subtype, cancer site, stage, and tumor grade for all histologic subtypes and age at diagnosis, cancer site, and tumor grade for the model restricted to advanced-stage HGS cancers. As shown in Table 4, among all histologic subtypes of epithelial ovarian cancer, having a diagnosis of primary peritoneal cancer was more associated with molecular signatures related to high stromal response (C1) and immune reaction (C2) clusters and less likely among the low stromal (C4) and mesenchymal/undifferentiated (C5) clusters ($P = 0.001$). These clusters were also more likely to have a diagnosis of the serous histologic subtype compared with the other four molecular signatures ($P < 0.0001$). Those with signatures consistent with the well-differentiated (C3), the low stromal response (C4), the mesenchymal/undifferentiated (C5), and the endometrioid (C6) clusters were more likely to have been diagnosed with a nonserous histologic subtype and less likely to have a diagnosis of primary peritoneal cancer. Poor tumor grade is consistent with clusters C1, C2, and C4, whereas well-differentiated tumors were observed more frequently in the C3 and C6 signatures ($P < 0.0001$). Similar patterns were observed restricting the analysis to the advanced-stage HGS cases. Advanced stage at diagnosis is positively correlated with the high stromal response (C1) cluster, and inversely associated with signatures consistent with the well-differentiated (C3) and the endometrioid (C6) clusters ($P = 0.006$). Heterogeneity in the age at diagnosis between the six signatures was also found for the combined histologic subtypes ($P = 0.0008$) and when the analysis was restricted to advanced-stage HGS ($P = 0.0161$). Older age at diagnosis was consistent with cancers that display the high stromal response (C1) and the mesenchymal/undifferentiated (C5) signatures, whereas an earlier age at diagnosis was consistent with the immune (C2), the

Table 2. Pearson correlation coefficients and (*P* values) for molecular clusters (C1–C6) and the TCGA survival signature in advanced-stage HGS ovarian cancers ($N = 106$)[a]

	C1	C2	C3	C4	C5	C6	TCGA survival signature
C1	1.00	−0.067 (0.497)	−0.049 (0.617)	−0.949 (<0.0001)	−0.505 (<0.0001)	−0.362 (0.0001)	−0.182 (0.062)
C2		1.00	−0.380 (<0.0001)	0.208 (0.033)	−0.710 (<0.0001)	−0.702 (<0.0001)	−0.153 (0.118)
C3			1.00	−0.055 (0.579)	0.110 (0.260)	0.463 (<0.0001)	−0.0228 (0.019)
C4				1.00	0.364 (0.0001)	0.201 (0.039)	0.158 (0.105)
C5					1.00	0.825 (<0.0001)	0.387 (<0.0001)
C6						1.00	0.220 (0.024)

[a]C1: high stromal response, C2: immunoreactive, C3: well differentiated, C4: low stromal response, C5: mesenchymal/undifferentiated, C6: endometrioid.

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Table 3. Frequency distribution of patient and tumor characteristics among invasive epithelial ovarian cancers and a subset of advanced-tage HGS ovarian cancer cases

Variable	Invasive ovarian cancer cases $N = 193$ n (%)	Advanced-stage HGS ovarian cancer cases $N = 106$ n (%)
Age, y		
0–49	38 (19.7)	15 (14.2)
50–59	67 (34.7)	40 (37.7)
60–69	62 (32.1)	38 (35.8)
≥70	26 (13.5)	13 (12.3)
Self-reported race		
Caucasian	156 (81.8)	91 (85.8)
African American	29 (15.0)	13 (12.3)
Other	8 (4.2)	2 (1.9)
Smoking status		
Never smoked	102 (52.85)	58 (54.72)
Current smoker	29 (15.03)	16 (15.09)
Ex-smoker	62 (32.12)	32 (30.19)
Diagnosed infertility		
Yes	22 (11.4)	12 (11.3)
No	171 (88.6)	94 (88.7)
Age at last pregnancy		
Never	28 (14.5)	13 (12.3)
<24	39 (20.2)	22 (20.7)
25–29	56 (29.0)	31 (29.3)
30–34	41 (21.2)	19 (17.9)
≥35	29 (15.0)	21 (19.8)
Number of live births, y		
None	35 (18.1)	18 (17.0)
1–2	95 (49.2)	53 (50.0)
3–4	55 (28.5)	31 (29.2)
≥5	8 (4.2)	4 (3.8)
Age at menarche		
<12	45 (23.4)	20 (18.9)
>12	147 (76.6)	86 (81.1)
Missing	1	
Menopausal status at diagnosis		
Premenopausal	50 (26.0)	25 (23.6)
Postmenopausal	142 (74.0)	81 (76.4)
Missing	1	
Ever diagnosed with endometriosis		
Yes	18 (9.4)	7 (6.6)
No	173 (90.6)	99 (93.4)
Missing	2	
Oral contraceptive use, y		
Never	76 (40.4)	38 (37.3)
<1	24 (12.8)	13 (12.7)
1–<5	42 (22.3)	25 (24.5)
≥5	46 (24.5)	26 (25.5)
Missing	5	4

(Continued on the following column)

Table 3. (Cont'd)

Variable	Invasive ovarian cancer cases $N = 193$ n (%)	Advanced-stage HGS ovarian cancer cases $N = 106$ n (%)
Breastfeeding (among ever pregnant)		
Never	90 (57.0)	52 (59.1)
<6 mo	33 (20.9)	17 (19.3)
6–12 mo	14 (8.8)	7 (8.0)
>12 mo	21 (13.3)	12 (13.6)
Tubal ligation		
Yes	52 (26.9)	32 (30.2)
No	141 (73.1)	74 (69.8)
BMI (kg/m^2) 1 y before diagnosis		
<25	75 (39.9)	45 (44.1)
25–<30	49 (26.1)	26 (25.5)
30–<35	29 (15.4)	17 (16.7)
>35	35 (18.6)	14 (13.7)
Missing	5	4
Family history of breast and ovarian cancers		
Yes	46 (23.8)	24 (22.6)
No	147 (76.2)	82 (77.4)
Peritoneal		
Yes	19 (9.8)	16 (15.1)
No	174 (90.2)	90 (84.9)
Histologic subtype		
Serous	126 (65.3)	106 (100)
Endometrioid	25 (13.0)	0 (0)
Mucinous	7 (3.6)	0 (0)
Clear cell	9 (4.7)	0 (0)
Other	26 (13.5)	0 (0)
Tumor grade		
Well differentiated	29 (15.1)	0 (0)
Moderate	67 (34.9)	54 (50.9)
Poor/undifferentiated	96 (50.0)	52 (49.1)
Missing	1	0
Tumor stage		
I/II	37 (19.2)	0 (0)
III/IV	156 (80.8)	106 (100)

well-differentiated (C3), and the low stromal response (C4) clusters.

Epidemiologic risk factors

Among all histologic subtypes combined (Table 5), after adjusting for age at diagnosis, the high stromal cluster (C1) was found to be inversely associated with a race of African American compared with non-African American, whereas those with molecular signatures consistent with the mesenchymal/undifferentiated (C5) and endometrioid (C6) clusters were more likely to be African American ($P = 0.008$). Women with well-differentiated (C3) and endometrioid (C6) type tumors were more likely

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Table 4. Multivariate mixed model tests for heterogeneity of relationships between molecular signatures[d] and age at diagnosis and tumor characteristics[e]

	C1		C2		C3		C4		C5		C6		
Variable	β	**SE**	β	**SE**	β	**SE**	β	**SE**	β	**SE**	β	**SE**	**Mixed model *P***
All invasive ovarian cancer cases													
Age at diagnosis, y													
20–49	Referent		Referent		Referent		Referent[b]		Referent		Referent		0.0008
50–59	0.153	0.184	−0.267	0.193	−0.065	0.154	−0.333	0.190	0.411	0.197	0.153	0.155	
60–69	0.052	0.188	−0.078	0.197	−0.256	0.157	−0.229	0.194	0.314	0.201	0.162	0.158	
≥70	0.575	0.238	−0.275	0.249	−0.032	0.198	−0.858	0.245	0.183	0.254	−0.068	0.200	
Histologic subtype													
Serous	Referent		Referent		Referent		Referent		Referent		Referent[c]		<0.0001
Clear cell	0.549	0.348	−0.630	0.365	0.723	0.290	−0.647	0.358	0.075	0.372	0.657	0.292	
Endometrioid	−0.346	0.210	−0.150	0.220	0.023	0.175	0.228	0.216	0.295	0.224	1.261	0.176	
Mucinous	−0.298	0.372	−0.160	0.390	−0.133	0.310	0.361	0.383	0.423	0.398	1.131	0.312	
Other	−0.247	0.212	−0.367	0.222	0.058	0.177	0.185	0.218	0.569	0.227	0.783	0.178	
Primary cancer site													
Ovary	Referent[b]		Referent		Referent		Referent[b]		Referent[b]		Referent		0.0011
Peritoneal	0.860	0.228	0.311	0.239	−0.243	0.190	−0.687	0.234	−0.784	0.243	−0.285	0.191	
Stage at diagnosis													
TNM stage I/II	Referent[a]		Referent		Referent		Referent		Referent		Referent[a]		0.0061
TNM stage III/IV	0.465	0.203	−0.081	0.213	−0.152	0.169	−0.270	0.209	−0.078	0.217	−0.375	0.170	
Tumor grade													
Poor/undifferentiated	Referent		Referent[c]		Referent[c]		Referent		Referent		Referent[b]		<0.0001
Moderately differentiated	0.023	0.157	−0.672	0.164	0.477	0.131	−0.157	0.161	0.365	0.168	0.225	0.132	
Well differentiated	0.029	0.217	−1.044	0.228	1.886	0.181	−0.454	0.224	0.091	0.232	0.703	0.182	
Advanced-stage HGS ovarian cancer cases													
Age at diagnosis, y													
20–49	Referent		Referent		Referent		Referent		Referent		Referent		0.0161
50–59	0.045	0.278	0.016	0.264	−0.049	0.186	−0.214	0.288	0.286	0.284	0.046	0.166	
60–69	0.056	0.279	0.188	0.265	−0.271	0.187	−0.239	0.289	0.157	0.285	−0.018	0.167	
≥70	0.436	0.355	−0.447	0.337	0.286	0.237	−0.828	0.367	0.356	0.362	0.316	0.212	
Primary cancer site													
Ovary	Referent[b]		Referent[a]		Referent		Referent[a]		Referent[b]		Referent[b]		0.0013
Peritoneal	0.830	0.257	0.568	0.244	−0.325	0.172	−0.589	0.266	−0.978	0.262	−0.487	0.154	
Tumor grade													
Poor/undifferentiated	Referent		Referent[b]		Referent[b]		Referent		Referent[a]		Referent[b]		0.0003
Moderately differentiated	−0.091	0.187	−0.703	0.177	0.482	0.125	−0.011	0.193	0.477	0.190	0.308	0.112	

Multivariable regression analysis significance levels: [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.0001$.

[d]C1: high stromal response, C2: immunoreactive, C3: well differentiated, C4: low stromal response, C5: mesenchymal/undifferentiated, C6: endometrioid.

[e]Regression coefficients are the expected difference in the signature, measured in SDs, associated with unit change in the covariate.

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to have reported a diagnosis of endometriosis, whereas the mesenchymal/undifferentiated (C4) tumors were inversely associated with endometriosis ($P = 0.056$).

We conducted the same analyses restricting the sample to advanced-stage HGS ovarian cancers ($n = 106$; Table 6). Similar to the finding for all histologic subtypes, African Americans were less likely to have a molecular signature consistent with the high stromal response (C1) and immune (C2) clusters but more likely to have a molecular signature consistent with the remaining four clusters ($P = 0.003$). Among the advanced-stage HGS cases, months of breastfeeding was found to be differentially associated with the molecular signatures, with an inverse relationship with the high-grade stromal (C1) and the immune (C2) clusters and a positive relationship with the low stromal response (C4), the mesenchymal/undifferentiated (C5), and the endometrioid (C6) clusters ($P = 0.024$). Family history of breast or ovarian cancer in a first-degree relative was more likely to be observed among those with signatures consistent with the high stromal response (C1) and the immunoreactive (C2) clusters, whereas less likely reported by those with molecular signatures consistent with the remaining four clusters (C3–C6; $P = 0.034$). In addition, a borderline statistical difference ($P = 0.071$) between molecular signatures was observed with oral contraceptive use. However, these relationships were not monotone with increasing duration of oral contraceptive use. No heterogeneity in the associations with the molecular signatures and endometriosis was detected among HGS cases. This is consistent with eliminating the low-grade and nonserous cancers from this analysis.

As race, family history, and age at diagnosis had significant and unexpected association with expression subtype in our data, we examined associations with race, BRCA1/2 mutation status, and age at diagnosis in the TCGA data (4). Of the 489 TCGA subjects with expression data, 434 were White, 22 were Black, and 33 were of another race. We found no evidence for association between race and the subtype signatures as we have defined them here or between race and the TCGA subtype designations. However, we did find evidence of association in the TCGA data between both the subtype signatures and TCGA subtype calls and presence of a germline BRCA1 or BRCA2 mutation. BRCA1/2 mutation carriers had higher cluster of high stromal content (C1) and immune (C2) signatures ($P = 0.014$ and 0.680, respectively) and lower cluster C3–C6 signatures ($P = 0.160$, 0.075, 0.088 and 0.008, respectively). These findings are consistent with our study showing that first-degree family history associated with clusters C1 and C2. In addition, the relationships we observed with age at diagnosis in the TCGA were similar to those in the NCOCS data. In particular, in the TCGA data, older age at diagnosis was associated with the C5 cluster ($P = 0.002$), whereas younger age at diagnosis was associated with the C2 ($P = 0.060$), C3 ($P = 0.012$), and C4 ($P = 0.145$) clusters.

The TCGA-derived survival signature

In Sfakianos and colleagues (7), we showed that the TCGA survival signature was significantly (HR, 2.7; $P = 0.0007$) associated with overall survival among the advanced-stage, high-grade NCOCS samples. Here, we considered whether the TCGA survival signature represents a distinct etiologic phenotype of ovarian cancer. Analyses of associations with known prognostic characteristics may help to characterize whether the TCGA survival signature is an independent predictor of survival or a culmination of known prognostic factors (see Supplementary Table S1). We conducted multiple linear regression analyses examining relationships between the TCGA-derived survival signature and epidemiologic risk factors and tumor characteristics. A diagnosis of an epithelial subtype other than a serous ovarian cancer and a poor/undifferentiated tumor grade were both positively associated with the TCGA signature. We did not observe a relationship with stage at diagnosis or tumor site and the TCGA signature although there was a positive trend with advanced stage at diagnosis. Most risk factors including age at diagnosis were not found to be significantly associated with the TCGA-derived survival signature. Among all histologic subtypes, weak associations were seen with early age at menarche (<12 years) and decreased duration of oral contraceptive and the survival signature. These relationships were attenuated when the analysis was restricted to the HGS cases.

Discussion and Conclusion

Our results are consistent with the tumor characteristics of these clusters as defined by Tothill and colleagues (3). We show that early stage at diagnosis and well-differentiated tumor grade is associated with the clusters classified with well-differentiated (C3) and endometrioid (C6) features. We also found the serous histologic subtype to be positively associated with the high stromal content (C1) signature and by an immune signature (C2) compared with nonserous histologic subtypes, whereas serous histology was inversely associated with molecular clusters with well-differentiated and endometrioid features compared with other histologic subtypes. A novel finding is that a higher fraction of primary peritoneal cancers are consistent with the high stromal content (C1) signature compared with primary ovarian cancers, both for all histologic subtypes combined and among advanced-stage HGS cases. Therefore, the differences noted between the subtypes and a diagnosis of primary peritoneal cancer was distinguished from the serous histologic subtype.

To our knowledge, this is the first study to examine correlations between ovarian cancer risk factors and molecular subtype signatures recently described by Tothill and colleagues (3) as well as the TCGA ovarian cancer survival signature (4). Of note, both age at diagnosis and race seem to differ according to the intrinsic clusters defined by Tothill and colleagues The age diagnosis of

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Table 5. Multivariate mixed model tests for heterogeneity of relationships between molecular signatures[c] and epidemiologic risk factors adjusted for age at diagnosis for 193 invasive epithelial ovarian cancers of combined histologic subtypes[d]

	C1		C2		C3		C4		C5		C6		
Variable	β	**SE**	β	**SE**	β	**SE**	β	**SE**	β	**SE**	β	**SE**	**Mixed model *P***
Race													
Non-African American	Referent[a]		Referent		Referent		Referent		Referent[b]		Referent[a]		0.008
African American	−0.440	0.196	−0.180	0.202	0.183	0.198	0.261	0.196	0.553	0.198	0.489	0.198	
Age at menarche, y													
≥12	Referent		Referent		Referent		Referent		Referent		Referent		0.611
0–<12	0.097	0.172	−0.200	0.173	−0.026	0.169	−0.161	0.170	0.217	0.174	0.094	0.174	
Menopausal status													
Premenopausal	Referent		Referent		Referent		Referent		Referent		Referent		0.176
Postmenopausal	−0.079	0.229	−0.189	0.232	−0.283	0.225	0.012	0.228	0.233	0.233	0.205	0.232	
Number of live births													
None	Referent		Referent		Referent		Referent		Referent		Referent		0.151
1–2	0.104	0.196	−0.159	0.200	−0.153	0.197	−0.033	0.194	−0.046	0.200	−0.045	0.199	
3–4	0.368	0.219	−0.136	0.224	−0.118	0.220	−0.298	0.217	−0.077	0.224	−0.155	0.223	
≥5	0.276	0.391	−0.370	0.401	−0.170	0.394	−0.118	0.388	0.027	0.401	0.260	0.399	
Age at last pregnancy, y													
25–29	Referent		Referent		Referent		Referent		Referent[a]		Referent[a]		0.253
14–24	−0.318	0.205	−0.322	0.209	−0.059	0.193	0.315	0.206	0.454	0.214	0.239	0.201	
30–34	−0.297	0.200	−0.196	0.204	0.009	0.189	0.367	0.201	0.267	0.208	0.144	0.196	
≥35	0.162	0.224	0.178	0.228	−0.264	0.211	0.009	0.225	−0.218	0.233	−0.425	0.220	
Breasfeeding, mo													
Never	Referent		Referent[a]		Referent		Referent		Referent[a]		Referent		0.380
<6	−0.171	0.207	0.185	0.202	−0.051	0.194	0.195	0.208	−0.089	0.203	−0.220	0.201	
6–12	−0.146	0.295	0.245	0.287	−0.009	0.277	0.197	0.297	−0.123	0.289	−0.202	0.286	
≥12	−0.441	0.241	−0.627	0.235	0.127	0.226	0.289	0.243	0.704	0.236	0.461	0.234	
Duration of oral contraceptive use, y													
Never	Referent		Referent		Referent		Referent		Referent		Referent		0.374
0–<1	0.097	0.229	0.554	0.231	−0.222	0.229	−0.006	0.227	−0.479	0.235	−0.377	0.233	
1–<5	0.082	0.188	0.112	0.190	−0.135	0.188	−0.029	0.187	−0.077	0.193	−0.360	0.191	
≥5	0.411	0.183	0.000	0.185	−0.131	0.183	−0.336	0.182	−0.102	0.188	−0.163	0.186	
Doctor diagnosis of infertility													
No	Referent		Referent		Referent		Referent[a]		Referent		Referent		0.460
Yes	0.426	0.222	−0.209	0.228	0.059	0.224	−0.477	0.220	−0.122	0.227	−0.143	0.227	

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Table 5. Multivariate mixed model tests for heterogeneity of relationships between molecular signatures[c] and epidemiologic risk factors adjusted for age at diagnosis for 193 invasive epithelial ovarian cancers of combined histologic subtypes[d] (Cont'd)

	C1		C2		C3		C4		C5		C6		
Variable	β	**SE**	β	**SE**	β	**SE**	β	**SE**	β	**SE**	β	**SE**	**Mixed model *P***
BMI (kg/m^2)													0.218
25–<30	Referent		Referent		Referent		Referent		Referent		Referent		
13–<25	0.284	0.180	0.206	0.182	−0.102	0.180	−0.155	0.179	−0.386	0.181	−0.309	0.179	
30–<35	0.160	0.230	−0.037	0.233	−0.016	0.230	−0.030	0.229	−0.088	0.232	−0.290	0.229	
≥35	0.245	0.219	0.095	0.221	−0.037	0.219	−0.166	0.218	−0.113	0.220	0.141	0.218	
Tubal ligation													0.270
No	Referent		Referent[a]		Referent		Referent		Referent		Referent		
Yes	0.062	0.160	0.342	0.161	−0.080	0.160	0.048	0.159	−0.277	0.162	−0.311	0.161	
Endometriosis													0.056
No	Referent		Referent		Referent		Referent		Referent		Referent		
Yes	−0.055	0.244	−0.116	0.249	0.195	0.242	−0.216	0.241	0.027	0.246	0.263	0.246	
Smoking status													0.833
Never smoked	Referent		Referent		Referent		Referent		Referent		Referent		
Current smoker	−0.051	0.206	0.015	0.210	0.278	0.205	0.049	0.204	−0.034	0.210	0.169	0.209	
Ex-smoker	0.116	0.160	−0.004	0.163	−0.027	0.159	−0.116	0.159	0.033	0.163	0.088	0.162	
Family history of breast or ovarian cancers													0.961
No	Referent		Referent		Referent		Referent		Referent		Referent		
Yes	0.037	0.165	0.018	0.168	0.046	0.165	−0.049	0.164	−0.002	0.168	−0.050	0.167	

Multivariable regression analysis significance levels: [a]$P < 0.05$, [b]$P < 0.01$.

[c]C1: high stromal response, C2: immunoreactive, C3: well differentiated, C4: low stromal response, C5: mesenchymal/undifferentiated, C6: endometrioid.

[d]Regression coefficients are the expected difference in the signature, measured in SDs, associated with unit change in the covariate.

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Table 6. Multivariate mixed model tests for heterogeneity of relationships between molecular signatures[c] and epidemiologic risk factors adjusted for age at diagnosis for 106 advanced-stage HGS ovarian cancers[d]

	C1		C2		C3		C4		C5		C6		
Variable	β	**SE**	β	**SE**	β	**SE**	β	**SE**	β	**SE**	β	**SE**	**Mixed model *P***
Race													
Non-African American	Referent[b]		Referent		Referent		Referent[b]		Referent[b]		Referent[b]		0.003
African American	−0.979	0.272	−0.461	0.278	0.115	0.197	0.864	0.279	1.112	0.281	0.606	0.165	
Age at menarche													
≥12	Referent		Referent		Referent		Referent		Referent		Referent		0.559
0–<12	−0.272	0.244	−0.261	0.239	−0.082	0.167	0.256	0.246	0.448	0.253	0.117	0.149	
Menopausal status													
Premenopausal	Referent		Referent		Referent		Referent		Referent		Referent		0.733
Postmenopausal	−0.015	0.276	−0.030	0.270	−0.298	0.187	0.037	0.279	0.154	0.289	0.013	0.168	
Number of live births													
None	Referent		Referent		Referent		Referent		Referent		Referent		0.344
1–2	0.065	0.260	−0.125	0.261	−0.242	0.180	0.051	0.264	0.119	0.274	−0.024	0.161	
3–4	0.150	0.289	−0.101	0.289	−0.043	0.200	−0.110	0.293	0.138	0.304	0.058	0.178	
≥5	1.197	0.538	0.102	0.539	−0.295	0.372	−0.987	0.547	−0.949	0.566	−0.396	0.332	
Age at last pregnancy, y													
25–29	Referent		Referent		Referent		Referent		Referent		Referent		0.534
14–24	−0.491	0.274	−0.347	0.270	−0.172	0.165	0.456	0.274	0.666	0.289	0.248	0.165	
30–34	−0.187	0.299	−0.097	0.295	−0.194	0.180	0.242	0.300	0.336	0.315	0.043	0.181	
≥35	0.143	0.276	−0.146	0.272	0.026	0.166	−0.133	0.276	−0.061	0.291	−0.009	0.167	
Breasfeeding, mo													
Never	Referent		Referent[b]		Referent		Referent		Referent[a]		Referent[a]		0.024
<6	−0.324	0.281	0.385	0.253	−0.095	0.173	0.323	0.287	−0.075	0.281	−0.133	0.160	
6–12	−0.226	0.411	0.117	0.370	−0.083	0.253	0.206	0.420	−0.016	0.411	−0.061	0.234	
≥12	−0.644	0.314	−0.957	0.283	0.098	0.193	0.351	0.321	1.063	0.314	0.549	0.178	
Duration of oral contraceptive use, y													
Never	Referent[a]		Referent		Referent		Referent		Referent		Referent		0.071
0–<1	−0.178	0.298	0.652	0.297	−0.233	0.212	0.210	0.301	−0.291	0.326	−0.112	0.188	
1–<5	−0.266	0.247	0.120	0.246	0.048	0.176	0.231	0.250	0.167	0.271	0.221	0.156	
≥5+	0.452	0.236	0.024	0.235	−0.228	0.168	−0.418	0.239	−0.059	0.259	0.071	0.149	
Doctor diagnosis of infertility													
No	Referent		Referent		Referent		Referent		Referent		Referent		0.736
Yes	0.428	0.296	0.002	0.292	0.029	0.204	−0.420	0.299	−0.341	0.311	−0.211	0.180	

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Table 6. Multivariate mixed model tests for heterogeneity of relationships between molecular signatures[c] and epidemiologic risk factors adjusted for age at diagnosis for 106 advanced-stage HGS ovarian cancers[d] (Cont'd)

	C1		C2		C3		C4		C5		C6		
Variable	β	**SE**	β	**SE**	β	**SE**	β	**SE**	β	**SE**	β	**SE**	**Mixed model *P***
BMI (kg/m^2)													
25–<30	Referent		Referent		Referent		Referent		Referent		Referent		0.221
13–<25	0.101	0.236	0.222	0.233	0.029	0.163	0.047	0.239	−0.379	0.251	−0.222	0.145	
30–<35	−0.315	0.295	0.309	0.292	0.096	0.204	0.541	0.299	−0.084	0.313	−0.028	0.181	
≥35	0.373	0.323	0.004	0.319	0.382	0.223	−0.194	0.327	−0.436	0.343	−0.113	0.198	
Tubal ligation													
No	Referent		Referent		Referent		Referent		Referent		Referent		0.435
Yes	0.250	0.212	0.340	0.206	−0.024	0.146	−0.191	0.215	−0.388	0.220	−0.132	0.129	
Endometriosis													
No	Referent		Referent		Referent[a]		Referent		Referent		Referent		0.311
Yes	0.230	0.378	0.009	0.371	−0.535	0.254	−0.307	0.382	0.068	0.396	−0.109	0.230	
Smoking status													
Never smoked	Referent		Referent		Referent		Referent		Referent		Referent		0.194
Current smoker	−0.178	0.272	0.361	0.264	0.019	0.186	0.233	0.274	−0.003	0.284	0.005	0.166	
Ex-smoker	0.087	0.214	−0.090	0.208	0.040	0.147	−0.097	0.216	0.217	0.224	0.051	0.130	
Family history of breast or ovarian cancers													
No	Referent		Referent		Referent		Referent		Referent		Referent[a]		0.034
Yes	0.361	0.221	0.291	0.217	−0.246	0.151	−0.240	0.225	−0.250	0.233	−0.272	0.133	

Multivariable regression analysis significance levels: [a]$P < 0.05$, [b]$P < 0.01$.

[c]C1: high stromal response, C2: immunoreactive, C3: well differentiated, C4: low stromal response, C5: mesenchymal/undifferentiated, C6: endometrioid.

[d]Regression coefficients are the expected difference in the signature, measured in SDs, associated with unit change in the covariate.

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the patient is a well-documented prognostic factor for ovarian cancer outcomes, with older age associated with worse overall survival and presumably indicative of more aggressive disease (11). We observed positive associations between older age at diagnosis and clusters C1 and C5, the clusters identified by Tothill and colleagues (3) to associate with the worst unadjusted survival prospects.

Although most of the established epidemiologic risk factors, including years of oral contraceptive use and pregnancy history, do not seem to be strongly differentially associated with the molecular clusters, a few associations were observed, particularly among the advanced-stage HGS cases. Overall, heterogeneity is found with the association between cluster and a diagnosis of endometriosis among the combined histologic subtype but not when the analysis was restricted to HGS cases. We did not have the power to determine whether this association was driven by the clear cell and endometrioid cancer subtypes. Heterogeneity in the associations between months of breastfeeding and family history of breast and ovarian cancers and the molecular clusters became evident when the analysis was restricted to advanced-stage HGS tumors. Although there have been reports of risk factors associations, such as smoking and endometriosis, that are specific to histologic subtypes of ovarian cancer (1, 2), these results suggest that molecular signatures of epithelial ovarian cancer may define etiologic pathways beyond that of histology alone.

There has been a growing realization that molecular subtypes of ovarian cancer do not define distinct disease entities as subtypes do with breast cancer but rather characterize distinct patterns of functionally significant molecular activities that may coexist in varying ratios across tumors of the same histologic classification (9, 10). Although classifying a tumor by its dominant signature ignores these complexities, one would expect to observe similar associations using this phenotype description, albeit with reduced power. To this end, we compared results of multinomial response models of a nominal classification of each tumor to those from the multivariate response models of the subtype signatures. We binned the tumors by assigning each to the cluster with the largest signature and used polytomous regression analysis to measure associations between the resulting designations and selected variables, including age and race in all samples and for family history among advanced-stage HGS samples. Although the associations observed in the multinomial models were nonsignificant, the directions and relative magnitudes of the individual effects were comparable as was expected.

We also examined relationships between the tumor and epidemiologic risk factors and the TCGA survival signature. Although this signature was derived from serous tumors, our findings suggest that the TCGA survival signature is even more consistent with other histologic subtypes compared with the serous cancers. Compared with a poorly differentiated cancer, having a well to moderately differentiated cancer was inversely associated, suggesting that molecular changes in the survival signature are the underlying components of tumor grade. A possible interpretation of our results is that these other prognostic factors are independent of the TCGA signature in the predication of survival/outcome. Further work is required to understand the implications of these findings, if confirmed.

Our results are consistent with the previous reports of the association of the molecular signatures with histology, grade, and stage. Although we report novel findings of relationships of tumor and epidemiologic characteristics indicative of heterogeneity between molecular signatures, the current study has several notable limitations. Because of the relatively small sample size and inherent uncertainty in defining the signatures in FFPE tissue, the power to detect associations is not optimal. Because we used existing characterization of the molecular signatures, derived mostly from the serous and endometrioid histologic subtypes, patient survival, and among mostly Caucasian subjects, it is possible that these previously defined signatures may not be indicative of etiologic processes or pathways or perhaps not representative of molecular signatures in non-White populations. A more direct approach for detecting molecular signatures indicative of etiologic pathways and those that represent other racial/ethnic groups would be to conduct a supervised analysis to determine whether molecular signatures can be found that are associated with exposure to risk factors, an important future direction of study. Only recently has the technology advanced to make this feasible in a population-based study where FFPE tissue would be necessary to use.

Another limitation of this study is that we were unable to conduct a systematic evaluation of our findings in an independent dataset, as we were unable to locate a study that paired the necessary epidemiologic risk factor with tumor assay data. However, we were able to evaluate associations between the subtype signatures and race, family history of breast or ovarian cancer (using BRCA1/2 germline mutation status as proxy), and age at diagnosis, using the recently published TCGA data (4) and found the same pattern of association that we observed with age at diagnosis and family history in the NCOCS data. Younger age at diagnosis was associated with the immunoreactive, the well-differentiated and the low stromal response signatures in both the TCGA data and the NCOCS data, whereas an older age at diagnosis was associated with the mecsenchymal/differentiated signature in both datasets. Those who reported a family history of breast or ovarian cancer in a first-degree relative were more likely to be classified as having a high stromal content or immunoreactive (C1 or C2). Ultimately, a validation study with a much larger sample size will be necessary to adjudicate the associations we observe in the current study.

In summary, this is the first report to investigate whether ovarian cancer risk factors are differentially

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associated with existing definitions of molecular signatures of epithelial ovarian cancer. Although we report novel findings, further work is needed to determine whether our results can be confirmed and whether a different approach to define clusters of molecular expression in tumor tissue may be more optimal for discerning subtypes associated with the pathogenesis of ovarian cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J.M. Schildkraut, E.S. Iversen, A. Berchuck, J.R. Marks
Development of methodology: E.S. Iversen, R. Whitaker
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.M. Schildkraut, R. Whitaker, R.C. Bentley, A. Berchuck
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.M. Schildkraut, E.S. Iversen, L. Akushevich, A. Berchuck, J.R. Marks
Writing, review, and/or revision of the manuscript: J.M. Schildkraut, E.S. Iversen, A. Berchuck, J.R. Marks
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Berchuck
Study supervision: J.M. Schildkraut, A. Berchuck

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Joellen M. Schildkraut, Edwin S. Iversen, Lucy Akushevich, et al.

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