Cyclin E Overexpression in Epithelial Ovarian Cancer Characterizes an Etiologic Subgroup

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Abstract

Background: **The objective of this study was to determine whether cyclin E overexpression defines an etiologically distinct subgroup of ovarian cancer.**
Methods: **We analyzed data from 538 epithelial ovarian cancer cases and 629 controls enrolled in a population-based case-control study. Cyclin E protein overexpression was assessed using immunohistochemistry. Case-control and case-case comparisons were done to evaluate the relationship between cyclin E overexpression and epidemiologic risk factors. Logistic regression models were used to estimate odds ratios (OR) and 95% confidence intervals (95% CI) while adjusting for potential confounders.**
Results: **Case-control comparisons showed ovarian cancers with and without cyclin E overexpression have different associations with several epidemiologic risk factors. A dose-response relationship was observed between lifetime ovulatory cycles (LOC) and ovarian cancer that overexpressed cyclin E [OR, 1.8; 95% CI, 1.1-3.0 for moderately high LOC (265-390 cycles) and OR, 2.7; 95% CI, 1.6-4.5 for high LOC (>390 cycles) compared with low LOC (<265 cycles)], but no relationship was seen with cancers that lacked overexpression. The most important components of the LOC variable contributing to the differences in the association with the cyclin E subgroups of ovarian cancer were months of oral contraceptive use and months pregnant.**
Conclusions: **Cyclin E overexpression is associated with a high number of LOC, largely influenced by oral contraceptive use and pregnancy. This suggests that cyclin E overexpression is a molecular signature characteristic of ovarian cancer cases that may arise via a pathway that involves ovulation-induced alterations.**
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Introduction

Advances in understanding the pathogenesis of epithelial ovarian cancer have come from studies of epidemiologic risk factors and molecular alterations. One of the leading theories regarding ovarian cancer etiology is the ''incessant ovulation hypothesis.'' Evidence from epidemiologic studies generally have been supportive of the relationship between high lifetime ovulatory cycles (LOC) and increased ovarian cancer risk (1). Conversely, factors that suppress ovulation and reduce LOC (that is, pregnancy, oral contraceptives, and breast-feeding) are protective (2). The biological mechanisms that underlie these various protective factors are not well understood but may involve effects on proliferation, inflammation, oxidative stress, mutation, and DNA repair in the ovarian epithelium as well as the actions of gonadotropins and steroid hormones.

Alterations of genes involved in growth, senescence, apoptosis, and DNA repair have been shown to play a role in the development of epithelial ovarian cancers (3). In this regard, more than half of invasive ovarian cancers harbor mutations in the *TP53* gene (4, 5). Point mutations in the *K-ras* and *B-raf* genes are frequent in low malignant potential (LMP) ovarian tumors (6). The heterogeneity with respect to the molecular alterations suggests that ovarian cancers may arise by way of different molecular pathways.

Cyclin E overexpression has been noted in breast cancer and other types of cancer and often has been associated with poor outcome (7). Cyclin E is a positive regulator of proliferation that acts by complexing with cdk2 to stimulate G_1-S-phase progression. In addition, there is evidence that cyclin E interacts with the centrosome and that overexpression leads to polyploidy, which may contribute to genomic instability (8, 9). Overexpression of cyclin E is one of the most frequent molecular alterations described thus far in advanced ovarian cancers (10-14). In a study of 134 advanced-stage, suboptimally debulked ovarian cancers treated on Gynecologic Oncology Group protocol 111, overexpression of cyclin E (>40% of cancer cells staining) was noted in 45% of cancers and was associated with significantly shorter survival (15). In a subset of 20 cases, amplification of the cyclin E gene was noted in 8 of

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10 cases with overexpression and in only 1 of 10 lacking overexpression.

The North Carolina Ovarian Cancer Study is a population-based case-control study that addresses epidemiologic risk factors, clinicopathologic characteristics, and molecular alterations (16). This molecular epidemiologic approach has the potential to elucidate the etiologic heterogeneity of ovarian cancer and to resolve distinct disease subsets based on risk factors and their corresponding molecular alterations. In view of the potential importance of cyclin E overexpression in the pathogenesis of a sizable fraction of ovarian cancers, we examined the relationship between this molecular alteration and epidemiologic risk factors and clinical features in the context of a population-based study. The goal was to determine whether cyclin E overexpression is a molecular signature of ovarian cancers that develop by way of an etiologic pathway that is characterized by specific underlying risk factors.

Materials and Methods

Subjects. Study subjects were enrolled through the ongoing North Carolina Ovarian Cancer Study, a population-based, case-control study of newly diagnosed epithelial ovarian cancer. Epithelial ovarian cancer cases were identified through the North Carolina Central Cancer Registry, a statewide population-based tumor registry, using rapid-case-ascertainment. Pathology reports for all ovarian cancer cases diagnosed in the study area were forwarded to the North Carolina Central Cancer Registry and then to the study office within 2 months of diagnosis. Eligibility criteria for ovarian cancer cases include diagnosis since January 1, 1999, ages 20 to 74 years at diagnosis, no prior history of ovarian cancer, and residence in a 48-county area of North Carolina. Data included in the current analyses are for subjects enrolled in phase I of the study with cancer diagnoses through March 2003. All participants were English speaking, mentally competent to complete an interview, and able to give informed consent. Physician permission was obtained before an eligible case was contacted. All cases underwent standardized pathologic and histologic review by the study pathologist to confirm diagnosis. Both invasive and LMP epithelial ovarian cancer cases were included. The response rate among eligible cases was 75%. Nonresponders were classified as patient refusal (7%), inability to locate the patient (9%), physician refusal (4%), death (4%), or debilitating illness (2%).

Population-based controls were identified from the same 48-county region as the cases and were frequency matched to the ovarian cancer cases based on race (African American or other, the majority of which were Caucasian) and age (5-year age categories) using list-assisted random-digit dialing. As required for the cases, controls had to be English speaking, mentally competent to complete an interview, and able to give informed consent. Potential controls were screened for eligibility and were required to have at least one intact ovary and no prior diagnosis of ovarian cancer. Seventy-three percent of controls identified by random-digit dialing who passed the eligibility screening agreed to be contacted and sent additional study information. Among those sent additional study information the response rate was 64%. Nonresponders were classified as refusal (27%) and unable to contact (9%). The study protocol was approved by the Duke University Medical Center Institutional Review Board and the human subjects committees at the North Carolina Central Cancer Registry and each of the hospitals where cases were identified.

Questionnaire Data. Trained nurse interviewers obtained written informed consent from study participants at the time of the interview, which was usually conducted in the woman's home. A 90-min questionnaire was administered to obtain information on known and suspected ovarian cancer risk factors, including family history of cancer in first- and second-degree relatives, menstrual characteristics, pregnancy and breast-feeding history, infertility, hormone use, and lifestyle characteristics, such as smoking, alcohol consumption, physical activity, and occupational history. A life events calendar, which marked significant life events including marriage and education, was used to improve recall of reproductive and contraceptive history. Additionally, to assist in recall, a pictorial display of oral contraceptives, hormone replacement therapy (HRT), and prescription and over-the-counter nonsteroidal anti-inflammatory drugs was provided. Anthropometric descriptors (height, weight, and waist and hip circumference) were measured, and a blood sample (30 mL) was collected during the interview.

Immunohistochemistry. We were successful in obtaining paraffin-embedded tissue for 95% of both the invasive and LMP ovarian cancer cases who enrolled in the study. Formalin-fixed, paraffin-embedded tissues were serially sectioned in 4- to 5-μm-thick sections, deparaffinized in three changes of xylene, and then rehydrated in graded alcohols. The slides were quenched for endogenous peroxidase with an aqueous solution of 3% hydrogen peroxide for 10 min. The slides then were placed in 0.01 mol/L citrate buffer (pH 6.0) and underwent heat-induced antigen retrieval for a total of 15 min in the Decloaking Chamber (Biocare Medical). The sections were rinsed in three washes of PBS, preincubated in Background Terminator (Biocare Medical) for 5 min, and then incubated in a humidity chamber with primary antibody overnight at 4°C. Cyclin E was detected using a mouse monoclonal antibody clone 13A3 (Vector Laboratories) at 1:100. The primary antibody detection was accomplished with the two-step HPR method of detection using the Universal 4plusHPR horseradish peroxidase kit (Biocare Medical). This procedure includes a 10-min incubation with a biotinylated, affinity-purified secondary antibody followed by a 10-min incubation with avidin DH (biotinylated horseradish peroxidase H complex). The slides were developed with the chromogen diaminobenzidine. Finally, the slides were counterstained with methyl green and dehydrated and coverslipped. An adjacent tissue section was stained with H&E to evaluate the histology and ensure that viable cancer was present. Slides were read by two reviewers and scored with respect to the fraction of cancer cells exhibiting staining for cyclin E and the intensity of the staining (0, none; 1, light; 2, moderate; 3, heavy). Differences between observers were resolved by consensus. Staining results were expressed on a scale of 0 to 3 based on the sum of the

products of the fraction (0-1.0) of cells stained at different intensities (0-3). Cyclin E protein overexpression was defined as a score of >0.4, which corresponds to any staining of >40% of cancer cells or a more intense staining of a smaller fraction of cells. This threshold was chosen based on data from a prior study of cyclin E in ovarian cancer conducted by the Gynecologic Oncology Group (15). In addition, we also did sensitivity analysis using other thresholds for cyclin E overexpression (intensity index >0, intensity index >0.2).

Statistical Analysis. Established ovarian cancer risks factors related to hormones, ovulation, reproductive history, and family history were available for the analyses. This includes years of oral contraceptive use, total months pregnant, months of breast-feeding (months of nursing before regularly adding formula or any other foods), infertility (unsuccessfully tried to get pregnant for >24 months and problem was not due to infertility in partner), age at menarche, months of irregular periods, age at menopause, prior hysterectomy (yes or no), age at hysterectomy, prior tubal ligation (yes or no), body mass index (BMI; kg/m^2) 1 year before diagnosis/interview, family history of breast or ovarian cancer in first-degree relatives (yes or no), and menopause status. A woman was classified as postmenopausal if her periods stopped naturally and her last period was at least 12 months before date of diagnosis/interview. If a woman began HRT before her periods stopped, she was classified as postmenopausal if she was on HRT for at least 2 years or if she thought she began menopause at least 4 years before the date of diagnosis/interview. If a woman's periods had stopped as a result of a hysterectomy without bilateral oophorectomy, she was classified as postmenopausal if she was at least 51 years old at diagnosis/interview, or if she was <51 years old at diagnosis/interview, she was considered postmenopausal only if at least 4 years had passed since she thought she started going through menopause. Finally, women whose periods had stopped as a result of radiation or chemotherapy also were classified as postmenopausal.

As we have described previously, a composite variable for LOC was computed (1). We determined the total number of ovulatory cycles by calculating the number of months between age at menarche and age at last period/menopause and then subtracting the total number of months pregnant, breast-feeding exclusively, amenorrheic following pregnancy, on oral contraceptives, on noncontraceptive hormones, and any other episodes of missed or irregular periods. Noncontraceptive hormone use generally occurred in women who reported taking menopausal hormones before their menstrual periods had ceased. These women reported having periods while taking the hormones, but the cycles would most likely have been anovulatory due to the hormones (17). LOC were then calculated by taking the total number of ovulatory months and dividing them by the average cycle length. Average cycle lengths were calculated based on women's self-report, taking into account variations throughout their reproductive lives.

t tests for continuous variables and χ^2 tests for categorical variables were used in the analysis of the bivariate distributions of the epidemiologic factors according to case-control status while stratifying cases according to the presence of cyclin E overexpression. We used multivariable unconditional logistic regression to calculate odds ratios (OR) and 95% confidence intervals (95% CI) for the relationship between the composite variable for LOC and the risk of two ovarian cancer

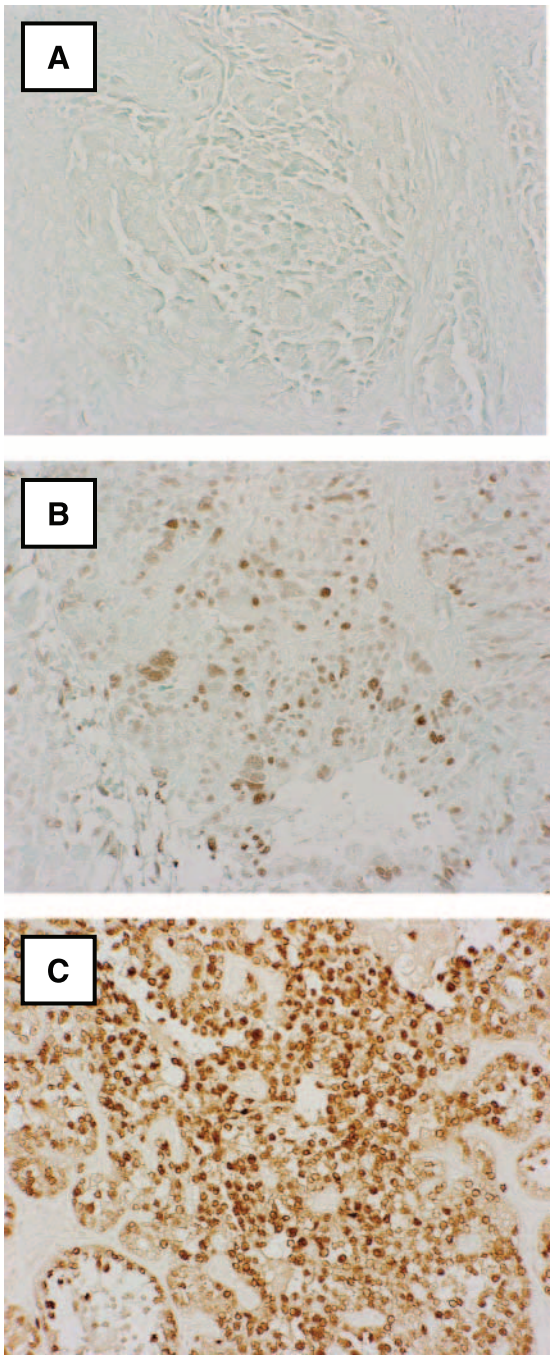

Figure 1. Cyclin E immunostaining of ovarian cancer (methyl green counterstain, ×200). **A.** No cyclin E expression. **B.** Cyclin E overexpression with 40% of cancer cells moderately staining. **C.** Cyclin E overexpression with 100% of cancer cells heavily staining.

subgroups defined by the presence of cyclin E overexpression. We adjusted the multivariable analyses for potential confounders including age at diagnosis/interview (included as cubic spline), race, infertility, prior tubal ligation, BMI 1 year before diagnosis/interview, and family history of breast or ovarian cancer in first-degree relatives. Additional analyses of ovarian cancer risk were conducted where the LOC composite variable was categorized based on tertiles of the distribution among controls. ORs and 95% CIs also were calculated for the case-case comparisons. In the case-case comparisons, ORs deviating from 1.0 suggest a possible heterogeneity between case subgroups. In the case-case models, stage (I/II versus III/IV) and grade (LMP, I/II, or III/IV) were included as potential confounders. Finally, we conducted additional subgroup analyses for invasive ovarian cancers and the subgroup of subjects who had not had a premenopausal hysterectomy since age at menopause would not have to be estimated in this subgroup. All analyses were done using SAS 9.1 (SAS Institute).

Results

Immunohistochemical detection of cyclin E protein in 413 invasive ovarian cancer cases yielded the following breakdown of expression: 37% had no detectable staining, 16% had staining in 1% to 20% of cells, 11% had staining in 21% to 40% of cells, 14% had staining in 41% to 70% of cells, and 22% of the cases had staining in 71% to 100% of cells. Figure 1 provides examples of cyclin E immunostaining. Overexpression of cyclin E was present in 183 of 413 (44%) of invasive ovarian cancers, including 168 with at least 40% of cells staining for cyclin E regardless of the intensity and 15 with <40% of cells stained but with moderate to heavy intensity.

Immunohistochemical detection of cyclin E protein in 123 LMP ovarian cancer cases yielded 54% with no detectable staining, 20% with staining in 1% to 20% of cells, 9% with staining in 21% to 40% of cells, 12% with staining in 41% to 70% of cells, and 4% of the cases with staining in 71% to 100% of cells. Overexpression of cyclin E was present in 34 of 123 (28%) of LMP cases, including 23 with at least 40% of cells staining for cyclin E regardless of the intensity and 11 with <40% of cells stained but with moderate to heavy intensity. The prevalence of cyclin E overexpression was significantly different in LMP (28%) compared with invasive ovarian cancers (44%; $P < 0.001$).

The relationship between cyclin E overexpression and clinicopathologic characteristics is shown in Table 1. The proportion of invasive cases that overexpressed cyclin E was higher among those with advanced stage at diagnosis (III/IV) compared with early-stage (I/II) cases (48% versus 38%, respectively). Differences according to histology and grade also were observed. Cyclin E overexpression was present in 46% of serous ovarian cancers, 84% of clear cell cases, and only 12% of the mucinous cases. Additionally, a larger proportion of higher-grade tumors showed cyclin E overexpression compared with grade 1 tumors. When considering the LMP cases, a somewhat smaller proportion of serous LMP cases overexpressed cyclin E compared with invasive cases (35% versus 46%, respectively). The proportion of mucinous LMP tumors that overexpressed cyclin E was the same (12%) as in the invasive cases. Because the distribution of cyclin E overexpression did not differ substantially within histologic subtype in invasive compared with LMP cases, we conducted combined analyses of these two types of ovarian cancer in addition to analyses restricted to invasive ovarian cancers.

The distributions of demographic features and epidemiologic risk factors in control subjects and ovarian cancer cases (invasive and LMP), stratified by cyclin E overexpression status, are shown in Table 2. Compared with controls, ovarian cancer cases that did not overexpress cyclin E were younger at diagnosis/interview. In unadjusted case-control comparisons, cases that overexpressed cyclin E showed risk factor associations consistent with the published literature, including an inverse relationship with duration of breast-feeding (18), pregnancies (19) and oral contraceptive use (20). In contrast, months of pregnancy was the only one of these three factors found to be significantly associated with

Table 1. Frequency of cyclin E overexpression in 413 invasive epithelial ovarian cancer cases and 123 ovarian cancer cases of LMP according to the tumor characteristics

	Invasive cases		LMP cases	
	No. cyclin E+/total (%)	*P*	No. cyclin E+/total (%)	*P*
All	183/413 (44)		34/123 (28)	
Stage				
Early (I and II)	49/129 (38)	0.063	28/96 (29)	0.539
Late (III and IV)	133/278 (48)		6/26 (23)	
Grade				
1	8/59 (14)	<0.001	NA	
2	52/119 (54)		NA	
3/4	122/226 (44)		NA	
Missing	1/9		NA	
Histology				
Serous	107/235 (46)	<0.001	29/84 (35)	0.018
Endometrioid	19/67 (28)		0/2 (0)	
Mucinous	3/26 (12)		4/34 (12)	
Clear cell	27/32 (84)		1/1 (100)	
Other	27/53 (44)		0/2 (0)	

ovarian cancers that lacked cyclin E overexpression. Additionally, a significant association with years of HRT and history of infertility was detected among cases that overexpressed cyclin E but not among those that lacked cyclin E overexpression. Higher BMI was associated with ovarian cancers that lacked cyclin E expression but not with those that overexpressed cyclin E. Cases that lacked cyclin E expression were more likely than controls to have undergone a hysterectomy, but no association with hysterectomy was detected among cases that overexpressed cyclin E. Finally, cases were less likely than controls to have had a tubal ligation regardless of cyclin E expression.

Multivariable logistic regression analyses of the relationship between the number of LOC and the risk of developing epithelial ovarian cancer (invasive or LMP) according to the presence of cyclin E overexpression are presented in Table 3. Controlling for potential confounders, we detected a significant relationship between the number of LOC and ovarian cancers that overexpress cyclin E but, there was no significant association with cancers that lacked cyclin E overexpression. For every additional 60 ovulatory cycles a woman experienced in her lifetime, the risk of cyclin E–positive ovarian cancer increased by ~18% (adjusted OR per 60 cycles, 1.18; 95% CI, 1.08-1.30). When analyses were restricted to invasive cancer only, the magnitude of the OR was somewhat stronger, showing a 23% increase risk for every additional 60 cycles (adjusted OR per 60 cycles, 1.23; 95% CI, 1.11-1.37; data not shown). The corresponding adjusted OR for the case-control comparison for cancers that lacked cyclin E overexpression was 1.05 (95% CI, 0.98-1.14; $P = 0.181$). Parallel analyses restricted to invasive ovarian cancer cases also did not support a relationship between LOC and invasive cancers that lacked overexpression of cyclin E (adjusted OR per 60 LOC, 1.04; 95% CI, 0.96-1.14).

Additional analyses were conducted with the individual components of the LOC composite variable simultaneously included in the model (see Table 3). The results suggest the main contributors to the differences in the relationship between the number of LOC and the risk of ovarian cancers with cyclin E overexpression were number of months pregnant and months of oral contraceptive use, which were found to be independent predictors when controlling for other component LOC variables and other potential confounders. In contrast, months pregnant and months of oral contraceptive use were not independently associated with ovarian cancer risk in the case-control analysis of component LOC variables among those cancers that lacked cyclin E overexpression. The only component of the LOC variable that appeared to be independently associated with the risk of ovarian cancer without cyclin E overexpression was months of HRT use where a positive association was found (OR, 1.04).

Additionally, the inclusion of other potential confounders in the multivariable analyses revealed that tubal ligation was an independent predictor of ovarian cancer risk regardless of cyclin E status. BMI was an independent predictor of ovarian cancer risk, although the association was not quite statistically significant for cases with cyclin E overexpression. Infertility was positively associated with cancers that overexpressed cyclin E, and a weaker nonsignificant association was found for cancers that lacked overexpression of cyclin E.

The relationships between LOC tertile and ovarian cancer stratified by cyclin E overexpression are presented in Table 4. In these analyses, the OR for being a cyclin E–positive case compared with a control was 1.8 for women with moderate number of LOC (265-390 cycles) and 2.7 for women with a high level of LOC (>390 cycles) compared with women with a low number of LOC (<265 cycles). When we stratified the cases and controls by menopausal status, this relationship was more pronounced in premenopausal women. Consistent with the results presented in Table 3, the distribution of LOC did not differ significantly between women with ovarian cancers lacking cyclin E overexpression compared with controls. Also presented in Table 4, the case-case comparisons found a significant association with LOC among ovarian cancers that overexpressed cyclin E compared with those that do not. Controlling for stage and grade as well as other potential confounders, the case-case comparisons revealed that women with higher numbers of LOC were more likely to have overexpressed cyclin E.

Sensitivity analysis was done using other thresholds of intensity >0 and >0.2 for cyclin E overexpression and an association was still noted between cyclin E overexpression and high LOC. Further analyses that restricted the study population to women who had not had a premenopausal hysterectomy resulted in very minimal changes to the OR estimates (data not shown).

Discussion

In the current study, we found a similar rate of cyclin E overexpression (44%) in invasive ovarian cancer as to that reported previously by Farley et al. (15). In this previous report, which included only patients with suboptimally debulked stage III/IV disease, there was no relationship between stage or histologic subtype and cyclin E overexpression. Among invasive ovarian cancer cases in our population-based study that included all stages at diagnosis, we did find a greater proportion of cyclin E overexpression among those diagnosed at an advanced stage, with serous or clear cell histology or with high grade. Similarly, among ovarian cancers of LMP, those of the serous histologic subtype were more likely to overexpress cyclin E. These observations are consistent with previous reports that found histologic subtypes of epithelial ovarian cancer may be etiologically distinct. Particularly, previously published reports support that the risk of mucinous ovarian cancers is associated with cigarette smoking (21-23) and endometrioid and clear cell cancers may arise from ovarian endometriosis or endometriomas (24, 25). These findings are in contrast to the findings among the most common serous histologic subtype of ovarian cancers, where no relationship was detected with these factors.

The case-control comparisons from phase I of the North Carolina Ovarian Cancer Study reveal that women with ovarian cancers that overexpress cyclin E had a greater number of LOC than controls. However, we did not find an association between the number of LOC and ovarian cancers that did not overexpress cyclin E. When the component variables for the number of LOC were

Table 2. Comparisons of demographic and epidemiologic characteristics between control subjects and ovarian cancer cases, stratified by cyclin E overexpression

Variables	Cyclin E+ cases (n = 218)	Cyclin E cases (n = 320)	Controls (n = 629)	Cyclin E+ cases vs controls	Cyclin E cases vs controls
				P	P
Age					
Mean (SD)	56.0 (11.6)	52.9 (11.5)	54.8 (12.2)	0.198	0.026
Median (range)	57 (21-74)	53 (20-74)	54 (20-75)		
Age (y) at menopause					
n	147	178	378		
Mean (SD)	49.2 (4.7)	49.2 (5.0)	48.9 (5.1)	0.569	0.587
Median (range)	50 (33-59)	50 (26-64)	50 (25-65)		
Age (y) at menarche					
Mean (SD)	12.6 (1.4)	12.5 (1.7)	12.6 (1.5)	0.976	0.212
Median (range)	13 (9-17)	12 (8-17)	13 (9-19)		
	n (%)	n (%)	n (%)		
Race					
White	192 (88)	262 (82)	529 (84)	0.181	0.619
Black	20 (9)	49 (15)	87 (14)		
Other	6 (3)	9 (3)	13 (2)		
Menopause status					
Pre/peri	71 (33)	140 (44)	250 (40)	0.060	0.236
Post	147 (67)	180 (56)	379 (60)		
Months breast-feeding					
None	166 (76)	221 (69)	405 (64)	0.006	0.120
1-5	29 (13)	59 (18)	117 (19)		
6-12	20 (9)	29 (9)	81 (13)		
>12	3 (1)	11 (3)	26 (4)		
Months pregnant					
None	37 (17)	52 (16)	61 (10)	0.003	0.002
1-8	10 (5)	21 (7)	23 (4)		
9-18	78 (36)	116 (36)	231 (37)		
19-36	77 (35)	109 (34)	252 (40)		
>36	15 (7)	21 (7)	61 (10)		
Years of oral contraceptive use					
None	87 (40)	104 (33)	203 (32)	<0.0001	0.862
≤5	94 (43)	114 (36)	239 (38)		
>5	34 (16)	92 (29)	178 (28)		
Unknown	3 (1)	10 (3)	9 (1)		
Irregular cycles (y)					
None	144 (68)	206 (68)	399 (65)	0.605	0.851
<3	38 (18)	55 (18)	114 (19)		
3-5	10 (5)	7 (2)	33 (5)		
>5	21 (10)	37 (120)	66 (11)		
Missing	5	15	17		
HRT (y)					
Never	110 (50)	190 (59)	375 (60)	0.005	0.223
<1	5 (2)	9 (3)	18 (3)		
1-<5	32 (15)	44 (14)	84 (13)		
5-<10	21 (10)	21 (7)	47 (7)		
>10	42 (19)	46 (14)	78 (12)		
Missing	8 (4)	10 (3)	27 (4)		
Hysterectomy					
No	168 (77)	228 (72)	493 (79)	0.757	0.048
Before menopause	45 (21)	81 (25)	124 (20)		
After menopause	5 (2)	9 (3)	10 (2)		
Hysterectomy but DK when	0	1	2		
Missing	0	1	0		
Family history of breast/ovarian cancer in a first-degree relative					
No	172 (79)	266 (83)	526 (84)	0.104	0.804
Yes	46 (21)	54 (17)	102 (16)		
BMI 1 y before diagnosis/interview (kg/m^2)					
<25	90 (42)	122 (39)	255 (42)	0.429	0.032
25-29.9	59 (28)	83 (27)	190 (31)		
≥30	65 (30)	107 (34)	166 (27)		
Missing	4	8	18		
Tubal ligation					
No	167 (77)	239 (75)	415 (66)	0.004	0.006
Yes	51 (23)	81 (25)	214 (34)		
Infertility					
No	164 (75)	261 (82)	526 (84)	0.004	0.366
Yes	54 (25)	59 (18)	101 (16)		

simultaneously included in the logistic regression model, months of oral contraceptive use and months pregnant were the only components of LOC found to be independent predictors of ovarian cancer that overexpressed cyclin E versus controls. It has been suggested that oral contraceptive use and pregnancy may prevent ovarian cancer both by suppression of ovulation and through an apoptotic effect due to exposure to progestin (26, 27). Additionally, case-case comparisons support the significant relationship between LOC and invasive ovarian cancers that overexpress cyclin E while controlling for tumor characteristics such as tumor behavior, stage, and grade. When analyses were restricted to invasive cancers, the relationship between LOC and cancers that overexpressed cyclin E became even stronger. However, there were too few LMP cases that overexpressed cyclin E ($n = 34$) to determine whether the association in LMP cases was significantly different from that among the invasive cases.

These data support the etiologic heterogeneity of ovarian cancer and suggest that various disease subsets arise through pathways that are characterized by distinct molecular signatures. Cyclin E overexpression may be indicative of differences in the etiologic pathways leading to the development of invasive ovarian cancer. The finding of a dose-response effect with respect to LOC suggests that this may be part of a causal pathway leading to this subgroup of cancers and further supports the incessant ovulation hypothesis. Perhaps cyclin E overexpression is a biomarker for proliferation of the ovarian epithelium resulting from exposure to greater numbers of LOC. There are several biological processes associated with ovulation that could lead to overexpression of cyclin E including increased proliferation to repair ovulatory wounds, oxidative stress, and inflammation as well as fluctuations in gonadotropin and steroid levels. However, our data do not allow us to distinguish the actual mechanisms that underlie the relationship between LOC and ovarian cancer that overexpress cyclin E.

Using a different study population, we reported previously that alterations in the *TP53* gene were positively associated with LOC and had suggested that this may be attributable to spontaneous mutations that occur with proliferation required to repair the ovarian surface during ovulation (4). This finding was not confirmed in a subsequent study (28). However, the current study, in which cyclin E overexpression was found to be associated with high LOC, supports the paradigm of the relationship between LOC and genomic instability. Further research of the relationship between cyclin E and p53 overexpression in ovarian cancer risk may help elucidate the molecular pathways involved in the relationship between ovulation and ovarian cancer risk.

Strengths of this study include the large study population from a well-designed case-control study with extensive epidemiologic data collected from an in-person interview. Due to the retrospective nature of the data collection, misclassification of some exposures is likely to have occurred, although differential misclassification by either cyclin E or case-control status is unlikely and therefore should not contribute to a spurious association. Although we were able to control for most known potential confounders, we could not rule out the possibility of residual confounding. One particular concern is that there may be residual confounding due to age at diagnosis/interview because age is correlated

Table 3. Multivariable analysis of the relationship between ovarian cancer risk according to the presence of cyclin E overexpression and composite LOC variable and components of LOC variable

	Cyclin E+ case versus control		Cyclin E- case versus control	
	OR* (95% CI)	*P*	OR* (95% CI)	*P*
Composite LOC variable (per 60 cycles)	1.18 (1.08-1.30)	0.001	1.05 (0.98-1.14)	0.181
Other covariates				
Tubal ligation (yes/no)	0.61 (0.42-0.89)	0.011	0.57 (0.41-0.80)	0.001
Family history of breast/ovarian cancer in first-degree relative (yes/no)	1.30 (0.94-1.79)	0.120	1.14 (0.83- 1.56)	0.413
BMI (kg/m^2)	1.02 (1.00-1.05)	0.128	1.02 (1.00-1.05)	0.030
Infertility (yes/no)	1.91 (1.29- 2.84)	0.001	1.26 (0.87-1.83)	0.227
LOC component				
Age at menarche (per year)	0.99 (0.88-1.11)	0.830	0.94 (0.86-1.03)	0.186
Age at last period (per year)	1.01 (0.97-1.05)	0.611	1.03 (0.99-1.07)	0.132
Breast-feeding exclusively (per year)	0.70 (0.41-1.21)	0.199	0.96 (0.65-1.40)	0.820
Pregnancy (per year)	0.81 (0.67-0.98)	0.026	0.89 (0.76-1.04)	0.148
Oral contraceptive use (per year)	0.91 (0.87-0.95)	<0.0001	0.99 (0.96-1.02)	0.318
Irregular cycles (per year)	0.97 (0.94-1.00)	0.074	0.98 (0.96-1.01)	0.203
Missed periods (per 12 periods)	1.02 (1.00-1.04)	0.076	1.00 (0.98-1.02)	0.984
HRT duration (per year)	1.02 (0.99-1.05)	0.187	1.03 (1.00-1.06)	0.022
Other covariates				
Tubal ligation (yes/no)	0.64 (0.42-0.98)	0.040	0.60 (0.42-0.85)	0.005
Family history of breast/ovarian cancer in first-degree relative (yes/no)	1.26 (0.90-1.76)	0.179	1.17 (0.85-1.60)	0.341
BMI (kg/m^2)	1.02 (1.00- 1.05)	0.067	1.03 (1.00- 1.05)	0.019
Infertility (yes/no)	1.80 (1.19- 2.73)	0.006	1.26 (0.85- 1.86)	0.247

NOTE: $n = 205$ cyclin E+ cases, $n = 166$ cyclin E-cases, and $n = 572$ controls.
*Adjusted for age at diagnosis/interview (cubic spline), race (Black/non-Black), BMI (kg/m^2; continuous), tubal ligation (yes/no), family history of breast/ovarian cancer in first-degree relative (yes/no), and infertility (yes/no).

Table 4. OR (95% CI) for case-control and case-case comparisons of the relationship between tertiles of LOC and cyclin E+ and cyclin E- ovarian cancer

	Cyclin E+ cases	Cyclin E-cases	Controls	Cyclin E+ cases versus controls	Cyclin E-cases versus controls	Cyclin E+ versus Cyclin E-cases
	n (%)	*n* (%)	*n* (%)	OR* (95% CI)	OR* (95% CI)	OR† (95% CI)
No. LOC						
All subjects						
<265	41 (19)	98 (32)	193 (32)	1.0	1.0	1.0
265-390	68 (32)	118 (39)	200 (33)	1.8 (1.1-3.0)	1.3 (0.9-1.9)	1.3 (0.7-2.2)
>390	104 (49)	89 (29)	219 (36)	2.7 (1.6-4.5)	1.0 (0.7-1.5)	2.4 (1.3-4.3)
Premenopausal						
<265	24 (34)	65 (48)	125 (51)	1.0	1.0	1.0
265-390	27 (39)	49 (36)	83 (34)	2.3 (1.1-4.8)	1.2 (0.7-2.1)	1.8 (0.7-4.2)
>390	19 (27)	22 (16)	38 (15)	5.2 (2.0-13.9)	1.4 (0.7-3.0)	3.5 (1.1-10.9)
Postmenopausal						
<265	17 (12)	33 (20)	68 (19)	1.0	1.0	1.0
265-390	41 (29)	69 (41)	117 (32)	1.4 (0.8-2.7)	1.3 (0.8-2.2)	1.0 (0.5-2.2)
>390	85 (59)	67 (40)	181 (49)	2.1 (1.1-3.8)	0.9 (0.5-1.5)	2.0 (1.0-4.1)

*Adjusted for age at diagnosis/interview (cubic spline), race (Black/non-Black), BMI (kg/m^2; continuous), tubal ligation (yes/no), family history of breast/ovarian cancer in first-degree relative (yes/no), and infertility (yes/no).
† Adjusted for age at diagnosis/interview (cubic spline), race (Black/non-Black), BMI (kg/m^2; continuous), tubal ligation (yes/no), family history of breast/ovarian cancer in first-degree relative (yes/no), and infertility (yes/no) as well as stage (I/II versus III/IV) and grade (LMP, I/II, or III/IV).

with the number of LOC. However, to avoid a spurious association due to confounding by age, we included age as a cubic spline variable in the logistic regression models. Analyses of the component variables for LOC are also consistent with our results of the relationship with LOC and ovarian cancer. It was also reassuring that the case-case comparisons, where we were able to control for tumor behavior, stage, and grade, were consistent with the case-control findings.

To our knowledge, this is the first report to examine the association between epidemiologic risk factors and cyclin E overexpression in ovarian cancer; therefore, confirmation of our findings in an independent study is needed. Our findings suggest that cyclin E overexpression is related to ovulation and that cyclin E plays a role in biological processes associated with proliferation and malignant transformation. *In vitro* experimentation has shown that cyclin E interacts with cdk2 and is involved in stimulating cell cycle progression. In addition, the cyclin E protein has a centrosomal localization sequence and is involved in regulating centrosome function at mitosis. Overexpression of cyclin E leads to chromosomal instability and aneuploidy via its interactions with the centrosome (9). Cyclin E in normally cycling cells is predominantly expressed in G_1-S but becomes ubiquitously expressed in some cancers. In addition, low molecular weight forms of cyclin E are produced by cleavage of the full-length 50-kDa protein by serine proteases (8). Some studies have suggested that the low molecular weight forms may be particularly oncogenic (7), but others have shown that these are simply related to overproduction of cyclin E (29). Although cyclin E overexpression may be due to gene amplification in some cancers, it also has been shown that the protein may be overexpressed due to cyclin E mRNA stabilization by the HuR regulatory protein (30).

The data presented in this article provide further support for the hypothesis that specific ovarian cancer risk factors may lead to the development of ovarian cancers that are characterized by distinct molecular signatures. Although age at menarche and menopause, pregnancy, oral contraceptive use, breast-feeding, and infertility all affect lifetime exposure to ovulation, these risk factors also have diverse effects on gonadotropin and steroid hormone levels. Some of these factors might affect risk independent of their effect on lifetime ovulations. It is possible that, in some cases, multiple etiologic risk factors and molecular alterations may contribute to the development of ovarian cancer. This certainly would be consistent with the observed heterogeneity of the disease pathologically and clinically. With the ability to gather both epidemiologic and molecular data, we can now seek to clarify these associations.

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