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PDF issue: 2025-12-05

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(Citation)
Cytopathology,29(3):254-261

(Issue Date)
2018-06

(Resource Type)
journal article

(Version)
Accepted Manuscript

(Rights)
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(URL)
https://hdl.handle.net/20.500.14094/90005192

Cleaved caspase-3 expression is a potential prognostic factor for endometrial cancer with positive peritoneal cytology

Running title: Cleaved caspase-3 in endometrial cancer

Word count: 2,470; Tables: 4; Figures: 3

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Acknowledgements: We thank Mitchell Arico and Angela Morben, DVM, ELS, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.
Conflict of interest statement: The authors disclose no potential conflicts of interest.
Disclosure of grants or other funding: This study was supported in part by a grant from the Grant-in-Aid for Scientific Research Program from the Japan Society for the Promotion of

Science (JSPS KAKENHI) [Grant Number: 15K08355].

Objective: Positive peritoneal cytology (PPC) in endometrial cancer remains a controversial topic. Cleaved caspase-3 (CC3) and Ki-67 are excellent markers of apoptotic and proliferating cells, respectively. The objective of this study was to determine the significance of CC3 and Ki-67 expression in peritoneal cytology samples as prognostic factors for endometrial cancer with PPC.

Methods: Sixty endometrial cancer specimens with PPC alone were divided into 51 endometrioid tumours (43 endometrioid carcinomas and 8 carcinomas with squamous differentiation) and 9 non-endometrioid tumours (2 serous carcinomas, 3 clear cell carcinomas, and 4 carcinosarcomas). CC3 and Ki-67 expression in peritoneal cytology samples were immunocytochemically assessed and correlated with disease-free survival (DFS) and overall survival (OS).

Results: Expression levels of CC3 and Ki-67 were not associated with any clinicopathological parameter. Patients with non-endometrioid tumours had significantly shorter DFS (P=0.001) and OS (P=0.001). Low CC3 expression ($CC3^{Low}$) was significantly associated with shorter OS (P=0.02), but not DFS (P=0.13). Multivariate analysis showed that non-endometrioid histology and $CC3^{Low}$ were independent prognostic factors. However, Ki-67 expression was not associated with survival. When endometrioid and non-endometrioid tumours were assessed separately, $CC3^{Low}$ was significantly associated with shorter DFS (P=0.002) and OS (P=0.002) in patients with non-endometrioid tumours.

Conclusions: Our results suggest that $CC3^{Low}$ in peritoneal cytology samples is a poor prognostic factor in patients with endometrial cancers, especially non-endometrioid tumours. Immunocytochemical analysis of CC3 expression could potentially facilitate identification of patients with high-risk endometrial cancer with PPC.

Keywords:, endometrial cancer, peritoneal washing cytology, immunocytochemistry, cleaved caspase-3, Ki-67

Introduction

Endometrial cancer is the most common malignancy of the female genital tract in developed countries, and its incidence is increasing.[1] The overall prognosis of women with endometrial cancer is favourable, but approximately 20% of patients will die of the disease. Analysis of factors to identify patients with more aggressive disease is one approach for individualized treatment. The appropriate decision for more extensive follow-up and aggressive treatment may reduce the mortality of endometrial cancer.

Endometrial cancers can spread into the abdominal cavity through the intact fallopian tube, resulting in extrauterine extension with positive peritoneal cytology (PPC) despite the absence of invasion of the serosa in the uterine body. In the International Federation of Gynecology and Obstetrics (FIGO) 1988 staging system, PPC was defined as one factor with which to determine stage IIIA. However, this definition was not included in FIGO 2008, which was revised for the first time in 20 years, because the independency of PPC *per se* as a prognostic factor had yet to be clarified.[2] Incidentally, PPC was considered a recommendation instead of a requirement in FIGO 2008. However, some reports indicate that PPC is a predictive factor for endometrial cancer.[3-6] Indeed, some cancers with PPC result in early relapse and death. These confounding issues have been in continuous debate even after updating the FIGO staging system.

Caspase-3 is a cysteine protease that plays a central role in execution of apoptosis.[7-9] It is synthesized as an inactive proenzyme, procaspase-3, which is activated by cleavage during apoptosis. The activated form is called cleaved caspase-3 (CC3) and plays pivotal roles in apoptosis induced through at least two pathways: the intrinsic (mitochondrial) pathway and extrinsic (death receptor-mediated) pathway. CC3 is responsible for specific cleavage of many key cellular proteins associated with apoptosis.[7-9] Polymorphisms and haplotypes of the *caspase-3* gene are risk factors for endometrial cancer.[10] However, although few immunohistochemical studies have assessed the association between the expression levels of

CC3 and survival of patients with cancer using an antibody specific for CC3 (unreactive to procaspase-3),[11-15] whether CC3 expression is involved in the prognosis of endometrial cancer remains unclear.

Ki-67 is commonly used as an excellent marker to define the proliferation status of tumour cells because its expression is found during all active phases of the cell cycle (G1, S, G2, and M) but is lost in the resting phase (G0).[16] However, the prognostic value of Ki-67 expression in endometrial cancer also remains controversial.[17-21]

Therefore, this study was conducted to assess the association between the prognosis of patients with endometrial cancer with PPC and the expression levels of CC3 and Ki-67 in peritoneal cytology samples as well as to clarify the prognostic significance of CC3 and Ki-67 expression.

Materials and Methods

Patients and tumours

This study was retrospective. We evaluated 60 patients with endometrial cancer who underwent total abdominal hysterectomy, salpingoophorectomy, or partial omentectomy with or without pelvic and/or para-aortic lymphadenectomy from 1990 to 2007 at the Kanagawa Cancer Center. The patients had little or no ascites, and peritoneal washing cytology was performed. The patient's tumours were classified as stage IIIA because of PPC alone according to FIGO 1988. However, they were all redefined as stage IA or IB according to FIGO 2008. All patients underwent postoperative chemotherapy. The clinicopathological findings of the patients and tumours are summarized in Table 1.

Preparation of peritoneal cytology samples for immunocytochemistry

We performed the cell decalcomania method (cell transfer method) using the following procedures. Papanicolaou-stained cytology samples were soaked in xylene to remove

coverslips. Adequate mounting medium (malinol; Muto Pure Chemicals, Tokyo, Japan) was added to the sample, followed by incubation at room temperature (RT) for 24 hours to set the mounting medium. The samples were then soaked in a water bath at 42°C for 1 hour, thereby softening the mounting medium. Immediately afterward, the mounting medium with the sample was peeled off. A portion was collected for immunostaining and transferred to another glass slide. Finally, the transferred samples were dried at 37°C for 24 hours and soaked in xylene, rehydrated in an ethanol gradient, and rinsed with tap water. Before immunostaining, samples were decolorized in 70% ethanol containing 1% hydrochloric acid.

Immunocytochemistry of CC3 and Ki-67

Following endogenous peroxidase blocking, heat-induced antigen retrieval was applied using a Pascal pressure chamber (Dako, Glostrup, Denmark) at 125°C for 1 minute in an optimal soaking solution: 1 mM ethylenediaminetetraacetic acid (pH 8.0) for CC3 or 10 mM citrate buffer (pH 6.0) for Ki-67. Cytology samples were incubated with primary anti-CC3 rabbit polyclonal antibody (1:400; Cell Signaling Technology, Danvers, MA, USA) or anti-Ki-67 mouse monoclonal antibody (clone MIB-1, 1:50; Dako) overnight at RT. After rinsing in 10 mM phosphate-buffered saline (pH 7.2), cell smears were incubated accordingly with either anti-mouse or anti-rabbit horseradish peroxidase polymer (Envision System; Dako) for 30 minutes at RT. The reaction products were visualized using a diaminobenzidine solution (Dako), and counterstaining was performed with haematoxylin.

Positive staining in more than 20% of tumour cells was considered ‘high’ expression ($CC3^{High}$ or $Ki\text{-}67^{High}$), and positive staining in less than 20% of tumour cells was considered ‘low’ expression ($CC3^{Low}$ or $Ki\text{-}67^{Low}$).

Statistical analysis

The Spearman rank correlation test was applied to assess the strength of correlations between

the expression levels of CC3 and Ki-67. Fisher's exact test was used to analyse the association between CC3 and Ki-67 expression and patient age, tumour size, histological type, depth of myometrial invasion, or vascular invasion. The disease-free survival (DFS) and overall survival (OS) rates were estimated using the Kaplan–Meier method, and differences between survival rates were compared by the log-rank test. DFS was defined as the time from the date of surgery to the date of first recurrence. The exact date of recurrence was defined as the date on which apparent tumours were detected by ultrasonographic or radiological examinations. OS was calculated from the date of surgery to the date of death. Data from the survivors were censored at the last follow-up. Multivariate analyses applying the Cox proportional hazards regression model were used to assess independency as a prognostic factor. Differences with a *P* value of <0.05 were considered statistically significant. All statistical analyses were performed using SPSS ver. 20 (IBM Corp., Armonk, NY, USA).

Ethical considerations

The study was approved by the Research Ethics Committee of Kanagawa Cancer Center. Informed consent was obtained from all patients.

Results

Association of CC3 and Ki-67 expression levels with clinicopathological parameters

The clinicopathological findings of the 60 patients and tumours are shown in Table 1. The median patient age was 57.0 years (range, 41–74 years); 14 (23%) were ≤50 years old and 46 (77%) were >50 years old. The median tumour size in the sections was 12.6 cm^2; 29 tumours (48%) were less than the median and 31 tumours (52%) were more than the median. We histologically divided the 60 endometrial cancer specimens into endometrioid and non-endometrioid tumours. The endometrioid tumours (n=51, 85%) included grade 1 endometrioid carcinomas (n=29), grade 2 endometrioid carcinomas (n=11), grade 3

endometrioid carcinomas (n=3), and adenocarcinomas with squamous differentiation (n=8). The non-endometrioid tumours (n=9, 15%) included serous carcinomas (n=2), clear cell carcinomas (n=3), and carcinosarcomas (n=4). The myometrial invasion depth was less than half of the myometrium for 41 tumours (68%) and more than half for 19 tumours (32%). Vascular invasion was positive for 24 tumours (40%) and negative for 36 tumours (60%).

Representative staining patterns of CC3 and Ki-67 in the peritoneal cytology samples are shown in Figure 1. $CC3^{High}$ was detected in 31 (52%) of the 60 samples, whereas 29 samples (48%) showed $CC3^{Low}$. Ki-67^{High} was observed in 33 samples (55%), whereas 27 samples (45%) showed Ki-67^{Low}. No significant correlation was found between the expression levels of CC3 and Ki-67 (r=−0.003, P=0.98).

The CC3 and Ki-67 expression levels were not correlated with any clinicopathological parameter including age, tumour size, histological type, depth of myometrial invasion, or vascular invasion (Table 2).

Association of CC3 and Ki-67 expression levels with patient survival in the cohort

The follow-up period for survivors ranged from 7 to 120 months with a median of 44.5 months. At the last follow-up, 87% of patients were still alive, 17% experienced tumour recurrence, and 13% had died of their disease. Table 3 shows the results of the univariate log-rank analysis of prognostic factors for DFS and OS in the cohort. As also shown in the Kaplan–Meier survival curves in Figure 2, patients with non-endometrioid tumours had significantly shorter survival than patients with endometrioid tumours (mean DFS, 33 vs, 108 months; P=0.001 and mean OS, 77 vs. 111 months; P=0.001). $CC3^{Low}$ was significantly associated with shorter OS (mean OS, 92 vs. 118 months, P=0.02), but not DFS (mean DFS, 93 vs. 108 months, P=0.13) compared with $CC3^{High}$. However, age, tumour size, depth of myometrial invasion, vascular invasion, and Ki-67 expression showed no significant associations with DFS or OS.

Table 4 shows the results of the multivariate Cox regression analysis of prognostic factors for DFS and OS in the cohort. Non-endometrioid histology (P=0.0001 for DFS and OS) and $CC3^{Low}$ (P=0.01 for DFS, P=0.005 for OS) were identified as significant independent prognosticators for shorter survival.

Association of CC3 expression levels with patient survival according to histological type

Kaplan–Meier survival curves and the results of the univariate log-rank analysis of DFS and OS according to histological type are shown in Figure 3 and Table 5, respectively. In the endometrioid group, patients with $CC3^{Low}$ tumours had significantly shorter OS than patients with $CC3^{High}$ tumours (mean OS, 102 vs. 120 months; P=0.04), whereas no significant association was detected between CC3 expression and DFS. Conversely, in the non-endometrioid group, patients with $CC3^{Low}$ tumours had remarkably shorter DFS (mean DFS, 7 vs. 47 months, P=0.002) and OS (mean OS, 14 vs. 111 months, P=0.002) than patients with $CC3^{High}$ tumours.

Discussion

The clinicopathological significance of PPC remains controversial because of its ambiguity as an independent prognostic factor.[3-6,22,23] Nevertheless, endometrial cancers with PPC occasionally have an unfavourable clinical course. Therefore, it is important to identify the prognostic factors influencing the survival of patients with endometrial cancer with PPC.

The clinicopathological parameters that affect the prognosis of endometrial cancers are widely known to include patient age, tumour size, histological type, depth of myometrial invasion, vascular invasion, extrauterine extension, lymph node metastasis, and distant metastasis.[1] In the present study, non-endometrioid histology was an independent factor for shorter DFS and OS with strong statistical significance, whereas no significant associations were found between the other variables and patient prognosis. Generally, non-endometrioid

carcinomas have different features and a worse prognosis compared with endometrioid carcinomas.[24-26] Additionally, some reports have indicated that grade 3 endometrioid carcinomas generally show aggressiveness and risk factors similar to those of non-endometrioid carcinomas.[22,23] Our results indicate that the histological type remains a prognostic factor for endometrial cancers even in a case group limited to endometrial cancers with PPC alone.

The prognostic value of CC3 expression in malignancies also remains controversial. CC3 expression is reportedly correlated with good prognoses in patients with gastric[12] and rectal[13] cancers or gliomas[11] but with poor prognoses in patients with pancreatic[14] or breast cancers.[15] To the best of our knowledge, no reports have yet addressed the prognostic significance of CC3 expression in endometrial cancers by immunostaining using a CC3-specific antibody. Our results showed that $CC3^{Low}$ in peritoneal cytology samples was an independent factor for shorter DFS and OS in patients with endometrial cancer with PPC. Downregulated expression of CC3 can render cancer cells resistant to apoptosis in response to extracellular apoptotic inducers including chemotherapeutic drugs and thus may affect the outcome and prognosis of the patient.[27]

The contribution of $CC3^{Low}$ to a poor prognosis was higher for patients with non-endometrioid tumours. In particular, we identified a subset of patients with a remarkably poor prognosis when they had $CC3^{Low}$ tumours: the mean DFS and OS of patients with non-endometrioid/$CC3^{Low}$ tumours were 7 and 14 months, respectively. These results suggest that non-endometrioid endometrial cancers with PPC showing $CC3^{Low}$ should probably be considered equivalent to advanced cancers because of their remarkably poor prognosis.

In contrast to CC3, Ki-67 expression in the peritoneal cytology samples was not associated with either DFS or OS. The clinical and prognostic significance of Ki-67 expression in endometrial cancers is also debateable. Some investigators have identified Ki-67 as an independent predictive factor for poor survival in patients with endometrial cancer,[18,19]

whereas others have indicated that Ki-67 is a predictor of survival in univariate analysis but not multivariate analysis.[17,20,21]

In conclusion, our results suggest that $CC3^{Low}$ in peritoneal cytology samples is a poor prognostic factor in patients with endometrial cancers, especially non-endometrioid tumours. Immunocytochemical analysis of CC3 expression could potentially facilitate identification of a subgroup of high-risk patients who should be considered equivalent to patients with advanced cancer. However, our results are still exploratory because the number of patients in this study is too small to reach any definitive conclusions. Further studies with a larger cohort are needed to validate our results.

In memoriam: This article is dedicated to the late Dr. Hiroki Nakayama (Head of the Department of Gynecology, Kanagawa Cancer Center) for his authentic and conscientious instructions throughout the course of this study.

Authors' contributions: N.O., Ma.Y., and S.K. designed the study, integrated the data, and wrote the manuscript. H.K. collected the clinicopathological data. N.O. and T.K. collected the tissue samples, prepared the paraffin-embedded sections, and performed the immunostaining and statistical analyses. Ma.Y., Mi.Y., and Y.K. performed the histopathological diagnoses. All authors read and approved the final manuscript.

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Table 1. Clinicopathological data of 60 patients with endometrial cancer with positive peritoneal cytology

Clinicopathological parameters	N
Age (years)	
≤50	14
>50	46
Tumour size (cm^2)	
≤12.6†	29
>12.6	31
Histological type	
Endometrioid	51
Endometrioid adenocarcinoma, grade 1	29
Endometrioid adenocarcinoma, grade 2	11
Endometrioid adenocarcinoma, grade 3	3
Adenocarcinoma with squamous differentiation	8
Non-endometrioid	9
Serous adenocarcinoma	2
Clear cell carcinoma	3
Carcinosarcoma	4
Depth of myometrial invasion	
Less than half	41
More than half	19
Vascular invasion	
Negative	36
Positive	24

†Median tumour size.

Table 2. Correlation between CC3 and Ki-67 expression levels and clinicopathological parameters

Variables	n	CC3			Ki-67		
		Low	High	*P-value*	Low	High	*P-value*
Age (years)							
≤50	14	7 (50)[†]	7 (50)	1.00	6 (43)	8 (57)	1.00
>50	46	22 (48)	24 (52)		21 (46)	25 (54)	
Tumour size (cm^2)							
≤12.6	29	12 (41)	17 (59)	0.32	10 (34)	19 (66)	0.13
>12.6	31	17 (55)	14 (45)		17 (55)	14 (45)	
Histological type							
Endometrioid	51	26 (51)	25 (49)	0.47	23 (45)	28 (55)	1.00
Non-endometrioid	9	3 (33)	6 (67)		4 (44)	5 (56)	
Depth of myometrial invasion							
Less than half	41	22 (54)	19 (46)	0.27	15 (37)	26 (63)	0.09
More than half	19	7 (37)	12 (63)		12 (63)	7 (37)	
Vascular invasion							
Negative	36	17 (47)	19 (53)	1.00	17 (47)	19 (53)	0.79
Positive	24	12 (50)	12 (50)		10 (42)	14 (58)	

CC3, cleaved caspase-3. [†]Number (%) of patients.

Table 3. Univariate analysis of prognostic factors for patient survival in the cohort

Variables	Mean DFS[†]	*P-value*[‡]	Mean OS[†]	*P-value*[‡]
Age (years)				
≤50	112	0.31	109	0.48
>50	97		105	
Tumour size (cm^2)				
≤12.6	99	0.95	103	0.45
>12.6	102		109	
Histological type				
Endometrioid	108	0.001*	111	0.001*
Non-endometrioid	33		77	
Depth of myometrial invasion				
Less than half	103	0.59	105	0.75
More than half	96		106	
Vascular invasion				
Negative	102	0.62	106	0.72
Positive	97		105	
CC3				
Low	93	0.13	92	0.02*
High	108		118	
Ki67				
Low	104	0.58	112	0.14
High	98		100	

DFS, disease-free survival; OS, overall survival; CC3, cleaved caspase-3. [†]Months, [‡]Log-rank test. *Statistically significant.

Table 4. Multivariate analysis of prognostic factors for patient survival in the cohort

Variables	DFS		OS	
	HR (95% CI)	*P-value*	HR (95% CI)	*P-value*
Histological type	18.89 (4.19–84.11)	0.0001*	29.90 (4.84–184.77)	0.0001*
CC3	0.13 (0.03–0.13)	0.01*	0.03 (0.003–0.37)	0.005*

DFS, disease-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; CC3, cleaved caspase-3. *Statistically significant.

Table 5. Univariate analysis of CC3 as a prognostic factor for patient survival according to histological type

Variables	Mean DFS†	*P-value*‡	Mean OS†	*P-value*‡
Endometrioid group				
$CC3^{Low}$	102	0.19	102	0.04*
$CC3^{High}$	116		120	
Non-endometrioid group				
$CC3^{Low}$	7	0.002*	14	0.002*
$CC3^{High}$	47		111	

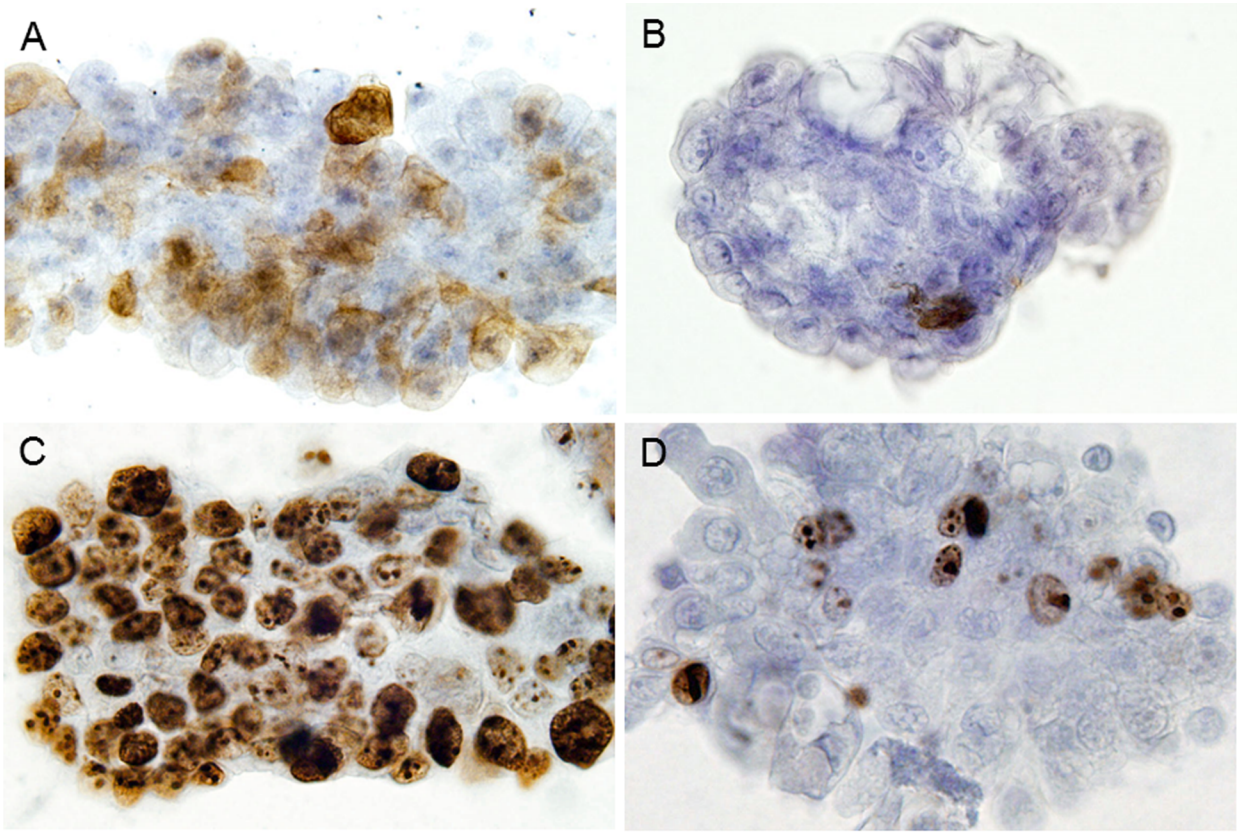
CC3, cleaved caspase-3; DFS, disease-free survival; OS, overall survival. †Months, ‡Log-rank test. *Statistically significant.

Figure Legends

Figure 1. Representative patterns of immunostaining for cleaved caspase-3 (CC3) or Ki-67 in peritoneal cytology samples of endometrial cancer. (A) High CC3 expression, (B) low CC3 expression, (C) high Ki-67 expression, (D) low Ki-67 expression. Strong immunoreactivities of (A) CC3 and (C) Ki-67 were observed in the cytoplasm and nuclei, respectively. Magnification, ×400.

Figure 2. Kaplan–Meier survival curves of patients with endometrial cancer with positive peritoneal cytology in the cohort. The histological type and expression of cleaved caspase-3 (CC3) were correlated with (A, C) disease-free survival (DFS) or (B, D) overall survival (OS). (A, B) Non-endometrioid histology was significantly associated with shorter DFS (P=0.001) and OS (P=0.001). (C, D) Low CC3 expression was significantly associated with shorter OS (P=0.02), but not DFS (P=0.13).

Figure 3. Kaplan–Meier survival curves of patients with endometrial cancer with positive peritoneal cytology in the (A, B) endometrioid group and (C, D) non-endometrioid group. Expression of cleaved caspase-3 (CC3) was correlated with (A, C) disease-free survival (DFS) or (B, D) overall survival (OS). (A, B) Low CC3 expression was significantly associated with shorter OS (P=0.04), but not DFS (P=0.19), in the endometrioid group. (C, D) In the non-endometrioid group, low CC3 expression was significantly associated with shorter DFS (P=0.002) and OS (P=0.002).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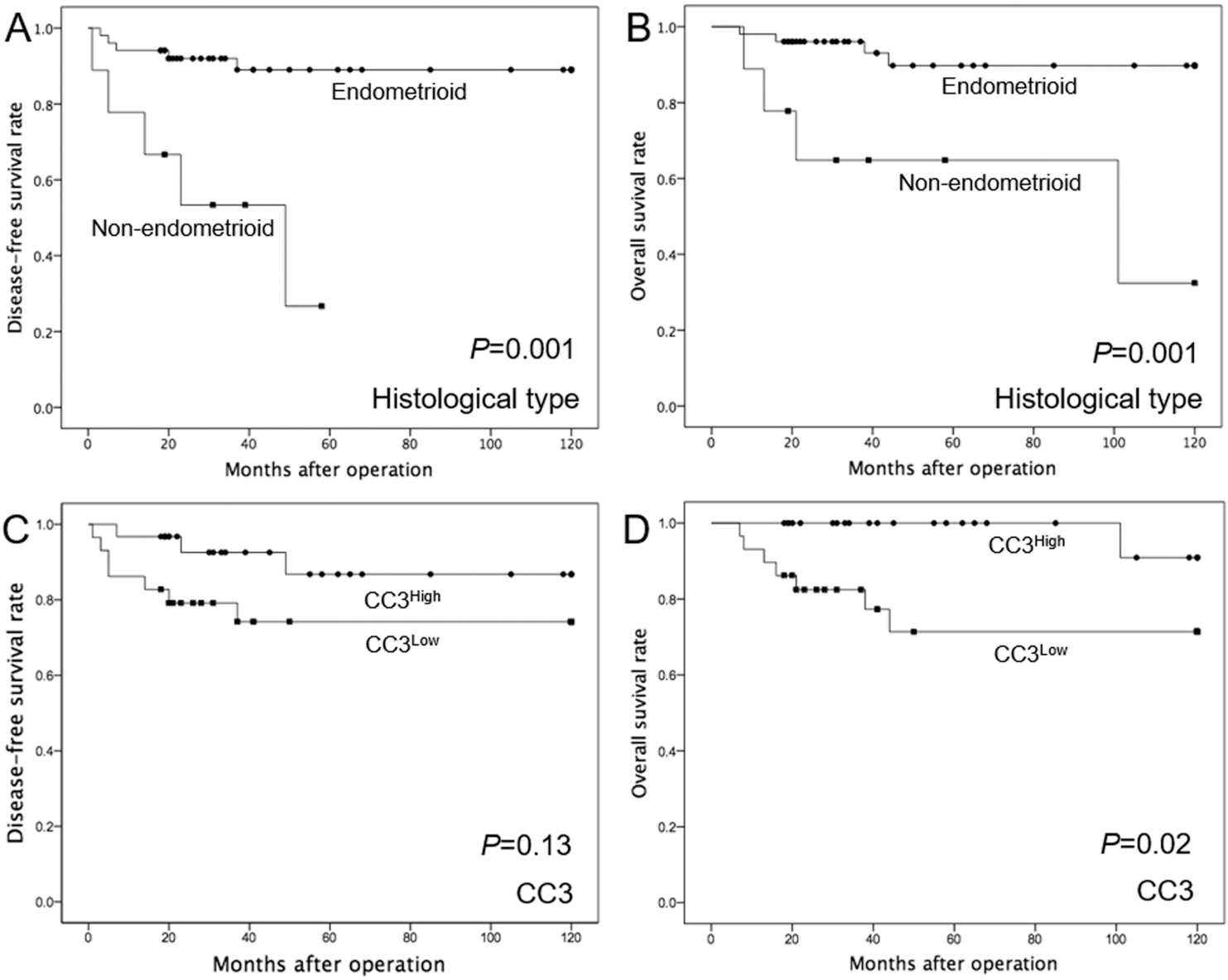
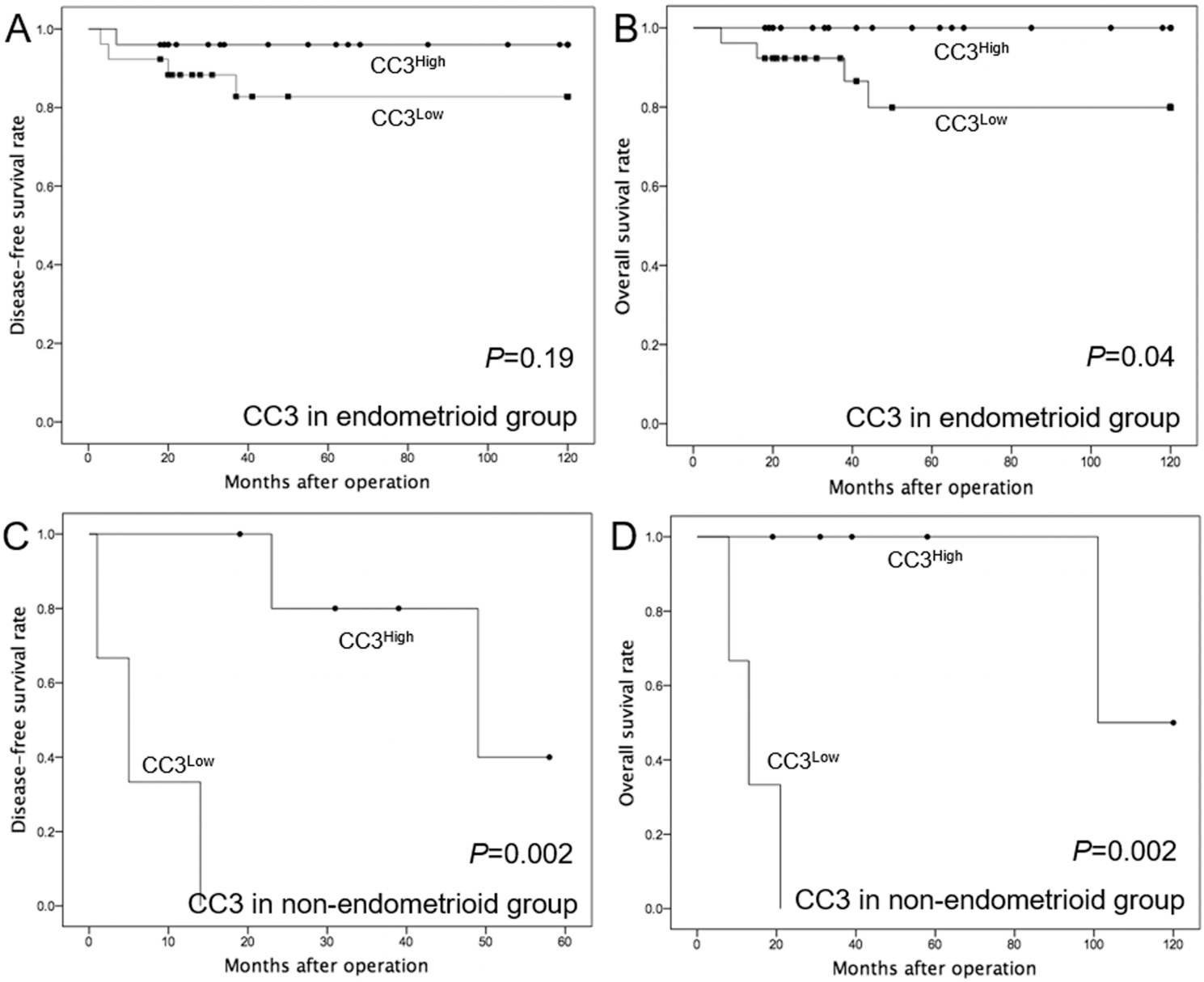
C
D


A
Disease-free survival rate
1.0
0.8
0.6
0.4
0.2
0.0
Endometrioid
Non-endometrioid
P=0.001
Histological type
0
20
40
60
80
100
120
Months after operation
B
Overall suvival rate
Endometrioid
Non-endometrioid
P=0.001
Histological type
Months after operation
C
Disease-free survival rate
CC3High
CC3Low
P=0.13
CC3
Months after operation
D
Overall suvival rate
CC3High
CC3Low
P=0.02
CC3
Months after operation


A
Disease-free survival rate
1.0
0.8
0.6
0.4
0.2
0.0
CC3High
CC3Low
P=0.19
CC3 in endometrioid group
0
20
40
60
80
100
120
Months after operation
B
Overall suvival rate
1.0
0.8
0.6
0.4
0.2
0.0
CC3High
CC3Low
P=0.04
CC3 in endometrioid group
0
20
40
60
80
100
120
Months after operation
C
Disease-free survival rate
1.0
0.8
0.6
0.4
0.2
0.0
CC3High
CC3Low
P=0.002
CC3 in non-endometrioid group
0
10
20
30
40
50
60
Months after operation
D
Overall suvival rate
1.0
0.8
0.6
0.4
0.2
0.0
CC3High
CC3Low
P=0.002
CC3 in non-endometrioid group
0
20
40
60
80
100
120
Months after operation