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Original Article

The Analysis of Cell Cycle–related Proteins in Ovarian Clear Cell Carcinoma Versus High-grade Serous Carcinoma

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Summary: In Japan, the frequency of ovarian clear cell carcinoma (CCC) is twice as high as that in the United States and Europe. Often, patient prognosis of CCC is poor because of chemoresistance. Here, we focus on the cell cycle, which is one of the mechanisms of chemoresistance. To detect the informative markers and improve the strategy of chemotherapy for CCC, we performed immunochemical staining of cell cycle–related proteins in ovarian malignant tumors. We detected that each of the 29 samples of CCC and high-grade serous carcinoma (HGSC) were necessary to reveal the significant differences in immunostaining and prognosis. We performed the immunostaining analysis using the antibodies of cell cycle–related proteins such as Ki-67, Cdt1, MCM7, and geminin. The positive rate of Cdt1 in the CCC group was significantly higher than that in the HGSC group ($P < 0.0001$). However, the positive rate of geminin in the HGSC group was significantly higher than that in the CCC group ($P < 0.0001$). The overall survival of CCC patients with high labeling index of Cdt1 was significantly worse than that of CCC patients with low labeling index of Cdt1 ($P = 0.004$). The study results suggested that the cancer cells of CCC and HGSC exist in the G1 phase and S, G2, and M phases, respectively. The differences in cell cycle of CCC might be one of the reasons for chemotherapy resistance. Further investigations are necessary to reveal the usefulness of Cdt1 as a biomarker in CCC. **Key Words:** Ovarian clear cell carcinoma (CCC)—Cell cycle—Cdt1—Geminin—Immunochemical.

Recently, the tailored medicine approach has been applied in several cancers, such as breast cancer, lung cancer, stomach cancer, and colon cancer, using pathologic segmentalization by immunochemical analysis. Although ovarian cancer is expected to respond to the postoperative adjuvant chemotherapy, there has not been sufficient progress in the field of tailored medicines.

Ovarian cancer, particularly surface epithelial ovarian carcinoma, is classified as either serous, mucinous, endometrioid, clear cell, or Brenner tumor. In Japan, clear cell carcinoma (CCC) is the second most common histologic subtype of epithelial ovarian carcinoma. The clinical behavior of CCC is distinctly different from that of other epithelial ovarian carcinoma subtypes (1). The current prevalence of CCC is 15% to 25%, with a reported increase from 19% to 24.5% during the period from 2002 to 2007 (2,3). CCC was diagnosed twice as frequently among Asian women living in the United States (11.1%), than among white women (4.8%) (2,4,5). About half of the

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cases of CCC (48.5%) are detected in the early stages (stages I or II) during initial diagnosis, while more than half of the cases of ovarian serous carcinoma (OSC) (61.7%) are detected in the advanced stage (stage III) (6). The overall survival (OS) of patients in stage III invasive CCC is worse than those in stage III invasive OSC. According to a study, the median OS of patients with CCC is only 24 mo compared with 45 mo for OSC (7). One of the reasons for this discrepancy is an extremely poor chemotherapy response rate (5,6,8). In addition, it has been reported that the progression-free interval of advanced CCC patients, whose first-line chemotherapy was effective, was within 6 wk (9). In addition, 2 of the 3 CCC patients who had disease recurrence died within 12 mo (10). Currently, there is no defined standard chemotherapy regimen that offers the best response to CCC. The Gynecologic Oncology Group (GOG) recommends that CCC treatment is completed using platinum-based chemotherapy, even during the early stages including stage I; however, there was no difference in the prognosis of stage IC CCC patients between those with and without chemotherapy (11). On the basis of these reports, it is difficult to determine how to apply chemotherapy in CCC patients.

We need to predict the effects of CCC chemotherapy, and set its adaptation criteria appropriately. The characteristics of CCC tumor cells compared with those of OSC tumor cells are: obviously few atypical mitotic figures, slow growth speed, and early stage (12). As most chemotherapy treatments suppress cell proliferation, it is possible to choose the correct target of therapy if we identify the characteristics of CCC cell cycle. The conventional classification of histopathologic tumor has limitations. We hypothesized that CCC tumor cells are readily present in G1 phase (G1 cell cycle arrest). The aim of this study is to propose a new CCC treatment strategy.

Cellular proliferation is regulated by the progression of the cell cycle. Cell cycle is divided into G0, G1, S, G2, and M (mitosis) phases. Cellular proliferation depends on the ability of the cell to successfully pass through the G1, S, G2, and M phases of the cell cycle. During microscopic analysis, cells with atypical mitotic figures can be observed only in the M phase. The M phase cells can be objectively detected by immunohistochemical analysis using the antibodies against proliferating cell antigens. In this study, we measured the labeling index (LI) of tumor cells using cell markers, which recognize different phases of cell proliferation. Through these experiments, we can understand the nature of the proliferation of tumor cells. It is well known that Ki-67 nuclear antigen is expressed in G1, S, G2, and M phases, but not in G0 phase (13–16). The Cdt1 and MCM7 nuclear antigens are mainly expressed in G1 phase (17,18) and G1 and S phases (19), respectively. Geminin, a nuclear antigen is expressed in S, G2, and M phases of the cell cycle (20). In this study, we investigated LI of the tumor cells of 2 types of ovarian cancers (CCC and OSC) using cell markers for immunostaining analysis. We also determined the relationships among the patterns of cell cycle protein staining, chemotherapy response rates and prognosis of these cancers.

MATERIAL AND METHODS

Study Population

In this study, we used ovarian tumor samples of CCC and high-grade serous carcinoma (HGSC) in Kawasaki Medical School Hospital between January 1990 and January 2016. We statistically analyzed the sample size as $\alpha = 0.05$, $1-\beta = 0.8$, $d = 15$ and $SD = 20$, and detected that each of the 29 samples of CCC and HGSC revealed significant differences in the results of immunostaining analysis and prognosis. We reanalyzed them histologically, and graded them using the Silverberg grading system (21). OSC has been classified into low-grade and high-grade types (22). In this study, we confirmed OSC of grades 2 and 3, based on the Silverberg grading system, as HGSC. Recently, FIGO (the International Federation of Gynecology and Obstetrics) and GOG grading system deleted the grading system of CCC (23). Hence, we did not use the grading system for CCC in our study. This study was approved by the local research ethics committee of Kawasaki Medical School and Kawasaki Medical School Hospital (No. 16-1280).

Immunohistochemical Analysis

Representative tissue blocks containing a sample of ovarian tumor were extracted from each patient. To determine the localization of cell cycle–related proteins in the ovarian cancer, formalin-fixed paraffin-embedded tissues were cut into silane-coated slides of 4-μm thickness. The slides were dewaxed in xylene and rehydrated through graded alcohols. We performed the immunostaining analysis using antibodies of cell cycle–related proteins, as demonstrated in Table 1, using a polymer-based Dako EnVison system technique (Dako Corp., Carpinteria, CA). For antigen retrieval, the sections were incubated in 0.01% (w/v) trypsin,

TABLE 1. *Immunohistochemistry Protocols*

Antigen	Clone	Dilution	Antigen-Antibody Reaction Time	Antigen Retrieval (Hot Bath 98°C)	Secondary Antibody
Ki-67	MIB-1	1:50	30 min	pH 9.0	Envision polymar
Geminin	Polyclonal	1:500	Over night	pH 6.0	
MCM7	Polyclonal	1:100	Over night	pH 6.0	
CDT1	Polyclonal	1:500	Over night	pH 9.0	
TP53	Polyclonal	1:50	60 min	pH 9.0	

including 0.01% $CaCl_2$ in 0.05 M Tris-buffered saline (pH 7.6) for 20 min at 37°C. The slides were then heated in a microwave in 0.1 M citrate buffer (pH 6.0) at 95°C for 20 min. Following antigen retrieval, endogenous peroxidase activity was quenched with 0.6% H_2O_2 in absolute methanol for 30 min at room temperature. The primary antibodies were applied as shown in Table 1. Antibodies were detected using the Envision polymer method (Dako Corp.) (24). The sections were counterstained with the Mayer hematoxylin. Known positive controls (ovarian serous adenocarcinoma for all) were also stained simultaneously.

Tumor Positive Cell Count Method and Interpretation

To determine the positive staining in each tumor, slides were evaluated in a low-power field (40×) to identify regions with the most intense staining. High-power fields (400×) were captured from the selected areas using a camera (OLYMPUS, Tokyo, Japan). Counts were performed using the software cellSens (OLYMPUS).

TABLE 2. *Patients' Characteristics (N = 58)*

	n (%)		
	CCC (n = 29)	HGSC (n = 29)	*P* (χ^2 test)
Age (median/range) (yr)	56 (21–75)	51 (28–67)	0.2899
FIGO stage*			<0.0001***
I	19 (65.5)	3 (10.3)	
Ia	7	0	
Ib	0	0	
Ic	12	3	
II	5 (17.2)	4 (13.8)	
III	4 (13.8)	17 (58.6)	
IV	1 (3.4)	5 (17.2)	
Grading†			
2	—	15 (51.7)	
3	—	14 (48.3)	
Recurrence			0.0347*
With	9 (31.0)	17 (58.6)	
Without	20 (69.0)	12 (41.4)	
Adjuvant chemotherapy			0.1501
Yes	27 (93.1)	29 (100)	
No	2 (7.4)	0	

CCC indicates clear cell carcinoma; HGSC, high-grade serous carcinoma.

More than 1000 cells were counted for each case. The positive rate was calculated by dividing the number of positive cells by the total number of cells counted.

TP53 Mutation

Immunostaining for p53 has been used as a surrogate marker for the presence of a TP53 mutation in HGSC. For statistical analysis, cutoff levels were stratified at 10% for p53. Positive judgment criteria for p53 were that the extent of staining was estimated to more than 10% level of positive tumor cells and the intensity of staining was more than moderate (25–28). Expression for p53 was examined for 29 HGSC cases to confirm that they are really high grade.

Statistical Analysis

OS was determined as the period between the first operative day of ovarian cancer and the day of death or the day on which patient's survival was confirmed. Disease-free survival was determined as the period between the first operative day of ovarian cancer and the day of recurrence of ovarian cancer or the day on which patient's survival was confirmed. We analyzed the prognosis in patients using the Kaplan-Meier

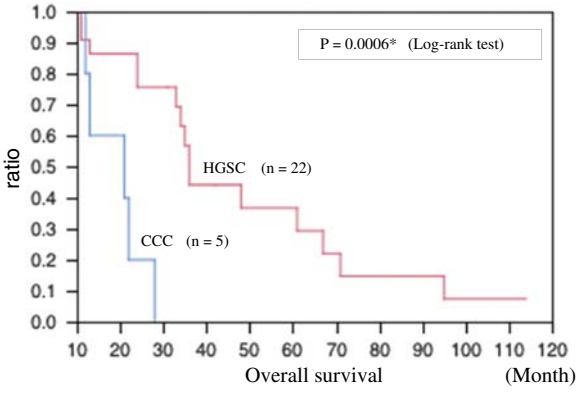


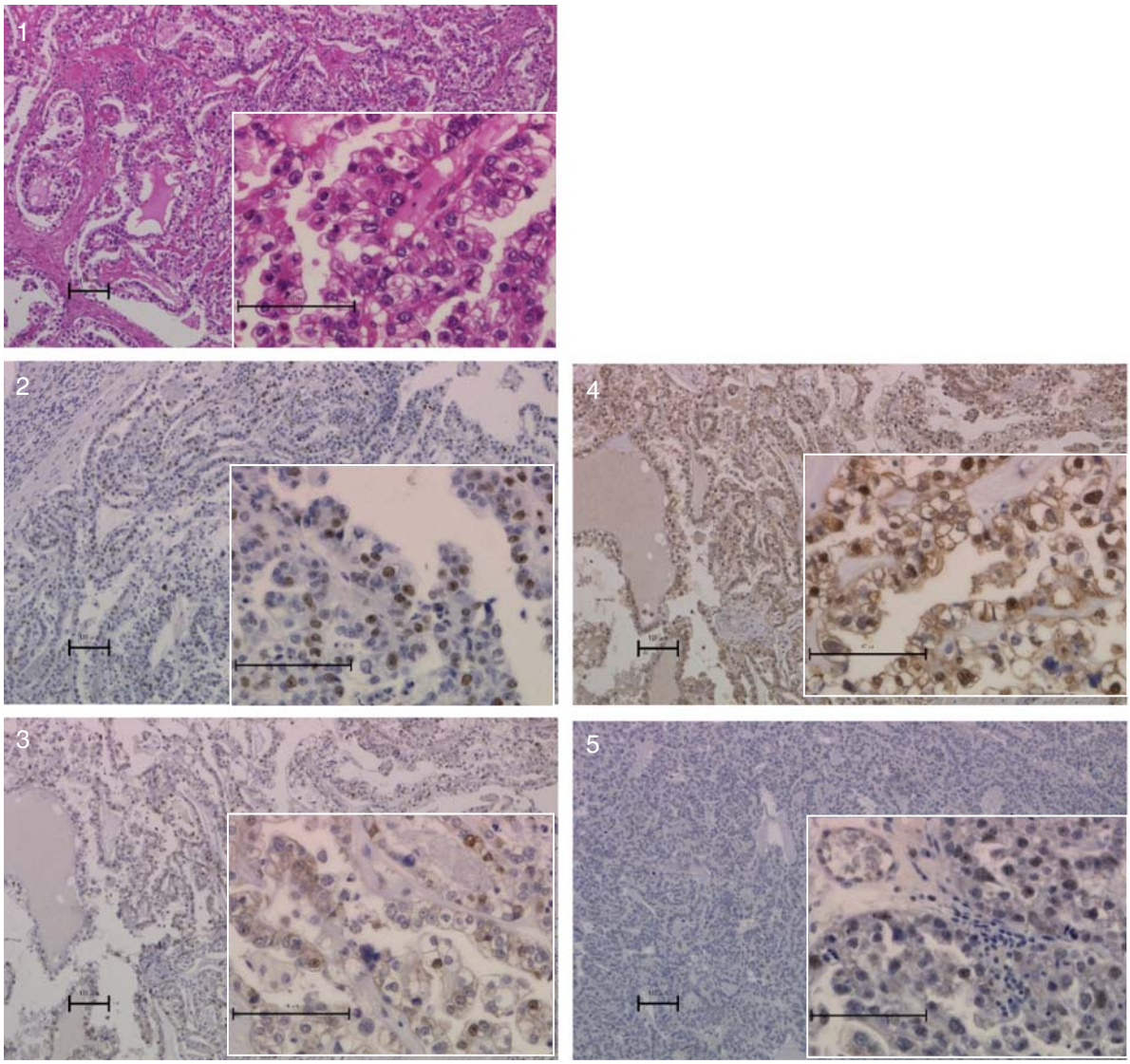
FIG. 1. Cumulative proportion surviving curve (Kaplan-Meier) of CCC and HGSC in the advanced stage. Overall survival was compared for patients with CCC and HGSC in stages 3 and 4. There was a significant difference in overall survival between the CCC group and the HGSC group ($P = 0.0006$). CCC indicates clear cell carcinoma; HGSC, high-grade serous carcinoma.

method. The Shapiro-Wilk W test, Mann-Whitney U test, and Student t test were used for statistical analysis, using the JMP version 9 program (SAS Institute Inc., Cary, NC). The values were represented as mean ± SD, and $P < 0.05$ was considered significant.

RESULTS

Patients' Characteristics and Outcomes

Patients' characteristics are demonstrated in Table 2. There was no significant difference in the mean age of patients between the 2 groups. The stages of the HGSC group were significantly worse than those of the CCC group. The recurrence rate of HGSC was also significantly higher than that of CCC. We compared the prognosis of HGSC patients and CCC patients in advanced stages. As shown in Figure 1, the OS of CCC patients was significantly shorter than that of HGSC patients, based on the results determined using the Kaplan-Meier methods ($P = 0.0006$). There was no significant difference in the disease-free survival between CCC patients and



AQ5 **FIG. 2.** Representative nuclear immunostaining patterns in tumor cells of clear cell carcinoma (CCC). (1) Hematoxylin and eosin–stained slides in tumor cells of CCC (original magnification, 100×, 400×). (2) Nuclear immunostaining with Ki-67 in tumor cells of CCC (original magnification, 100×, 400×). (3) Nuclear immunostaining with geminin in tumor cells of CCC (original magnification, 100×, 400×). (4) Nuclear immunostaining with MCM7 in tumor cells of CCC (original magnification, 100×, 400×). (5) Nuclear immunostaining with Cdt1 in tumor cells of CCC (original magnification, 100×, 400×).

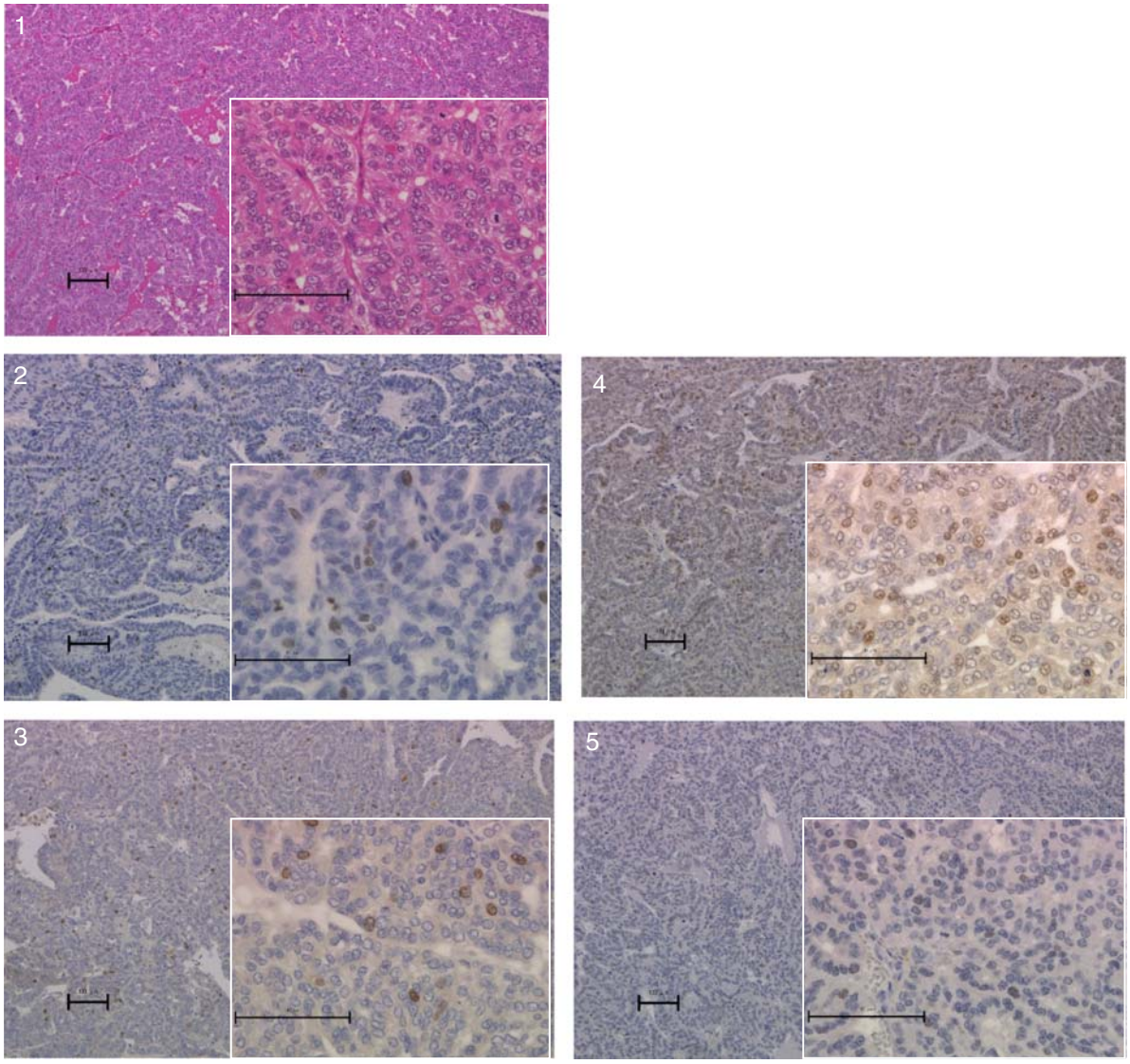


FIG. 3. Representative nuclear immunostaining patterns in tumor cells of high-grade serous carcinoma (HGSC). (1) Hematoxylin and eosin–stained slides in tumor cells of HGSC (original magnification, 100×, 400×). (2) Nuclear immunostaining with Ki-67 in tumor cells of HGSC (original magnification, 100×, 400×). (3) Nuclear immunostaining with geminin in tumor cells of HGSC (original magnification, 100×, 400×). (4) Nuclear immunostaining with MCM7 in tumor cells of HGSC (original magnification, 100×, 400×). (5) Nuclear immunostaining with Cdt1 in tumor cells of HGSC (original magnification, 100×, 400×).

HGSC patients. There was no significant difference between Cdt1 and chemotherapy in the CCC group in the χ^2 test ($P = 0.22$). There was also no significant difference in the HGSC group ($P = 0.96$).

Results of Immunohistochemical Analysis

We examined the hematoxylin and eosin staining and immunostaining results of geminin, MCM7, Cdt1, and p53. As shown in Figures 2 and 3, anti-geminin was stained in the HGSC group, and anti-Cdt1 in the CCC group. Tables 3 and 4 demonstrates the positive cell count results in the CCC and HGSC groups. All of HGSC patients were positive of p53 as stronger than (++). Figure 4 demonstrated the difference in positive rate of cell markers in both groups. The positive rate of Cdt1 in CCC group was significantly higher than that in the HGSC group ($P < 0.0001$). However, the positive rate of geminin in the HGSC group was significantly higher than that in the CCC group ($P < 0.0001$). There was no significant difference in the positive rates of Ki-67 and MCM7

TABLE 3. *Results of Tumor Cell Count of Clear Cell Carcinoma*

No.	Ki-67	Cdt1	MCM7	Geminin
1	55.0	67.0	45.8	14.6
2	49.4	66.6	59.6	15.2
3	63.6	69.2	52.4	19.1
4	55.4	70.9	40.0	11.1
5	21.1	82.2	38.9	6.6
6	34.0	67.9	35.5	8.2
7	27.0	64.1	41.0	9.1
8	19.1	55.2	41.5	9.2
9	52.5	75.9	38.9	10.3
10	49.1	76.2	41.3	5.7
11	73.5	63.8	42.1	11.6
12	37.4	53.0	64.1	19.5
13	15.8	69.2	56.6	12.8
14	32.2	57.1	45.1	11.5
15	77.8	60.3	50.5	17.6
16	45.0	53.9	55.7	15.1
17	45.1	66.4	48.0	16.7
18	21.9	64.1	44.6	21.3
19	49.4	59.0	42.0	19.8
20	13.4	39.1	44.3	10.6
21	50.4	43.1	50.9	14.9
22	37.1	43.2	56.6	8.9
23	65.7	54.0	46.5	16.8
24	24.3	44.8	38.9	22.9
25	51.0	43.7	52.7	12.1
26	43.2	53.9	43.3	6.1
27	5.6	52.6	39.4	8.1
28	38.7	60.1	54.8	13.0
29	66.9	48.2	47.6	20.5
Mean (SD)	42.1 (3.3)	59.5 (1.8)	46.8 (1.9)	13.4 (1.1)

TABLE 4. *Results of Tumor Cell Count of High-grade Serous Carcinoma*

No.	Ki-67	Cdt1	MCM7	Geminin
1	45.7	7.3	44.9	20.8
2	23.6	23.5	47.2	11.7
3	69.3	9.8	32.6	34.9
4	63.7	23.5	59.2	28.7
5	54.8	22.2	42.5	30.0
6	36.6	23.4	56.5	15.8
7	14.9	11.2	55.6	18.2
8	55.4	31.7	54.1	22.9
9	73.2	34.7	65.1	27.6
10	28.2	17.1	42.4	19.8
11	58.2	25.7	72.4	18.3
12	65.3	20.1	57.9	33.3
13	25.2	26.4	67.7	27.9
14	40.4	30.6	53.9	29.3
15	32.9	20.1	71.5	26.4
16	27.2	26.8	25.1	26.7
17	49.8	19.6	33.9	24.9
18	65.2	31.4	39.5	27.0
19	52.9	15.9	48.8	31.3
20	51.1	8.5	64.7	26.9
21	12.9	11.8	46.6	20.0
22	65.3	27.1	41.9	32.9
23	45.3	15.3	54.0	31.3
24	53.7	16.9	42.3	20.8
25	51.6	13.8	68.1	21.5
26	24.0	15.5	63.6	28.2
27	37.7	4.6	67.1	15.9
28	48.9	17.0	43.5	39.1
29	43.0	7.5	55.7	30.0
Mean (SD)	45.4 (3.3)	19.3 (1.8)	52.4 (1.9)	25.6 (1.1)

between the 2 groups. The study results suggested that the cancer cells of CCC and HGSC exist in G1 phase and S, G2, and M phases, respectively.

The Association Between OS and the Expression of Cdt1 in CCC

We examined the possibility that Cdt1 might be used as a biomarker for predicting the prognosis in CCC patients. We performed the Shapiro-Wilk W test and confirmed normal distribution (mean, 60.2; 95% confidence interval, 55.4–64.4; $P = 0.84$). We determined the cutoff point as 64% of Cdt1 positive rate. Figure 5 demonstrates the OS of CCC patients in stages higher than stage 2. The OS of CCC patients with a high positive rate group of Cdt1 was significantly worse than that of CCC patients with a low positive rate group of Cdt1 ($P = 0.004$). These results signify that CCC patients whose cancer cells were mainly present in G1 phase had poor prognosis. We also compared the OS of CCC patients in stages higher than stage 3; however, we could not obtain significant results because of the small sample size (n = 5).

DISCUSSIONS

Several previously reported mechanisms of drug resistance in the chemotherapy of ovarian cancer may be related to factors, such as decrease in the accumulation of drugs (29), inactivation of drugs (30,31), DNA repair (32), signal transduction pathway of cell growth factors (33–35), and cell cycle regulation. Cell growth is regulated by the cell cycle that includes G1, S, G2, and M phases. A cell cycle is controlled by cyclin-dependent kinase (CDK) and regulated by CDK inhibitors at 4 checkpoints(G1/S-phase, S-phase, G2/M-phase, and M-phase checkpoints). Itamoch et al. (36) demonstrated that CCC cells had lower CDK2 activity and higher p27 expression than serous adenocarcinoma cells. Reduced CDK2 activity via the cytoplasmic sequestration of CDK2 by p27 might contribute to the suppression of cellular proliferation and lead to chemoresistance in CCC (36).

Cdt1 is an essential component for the assembly of a prereplicative complex. Cdt1 activity is inhibited by geminin, which also participates in neural development and embryonic differentiation in many eukaryotes (37). Geminin prevents replication before

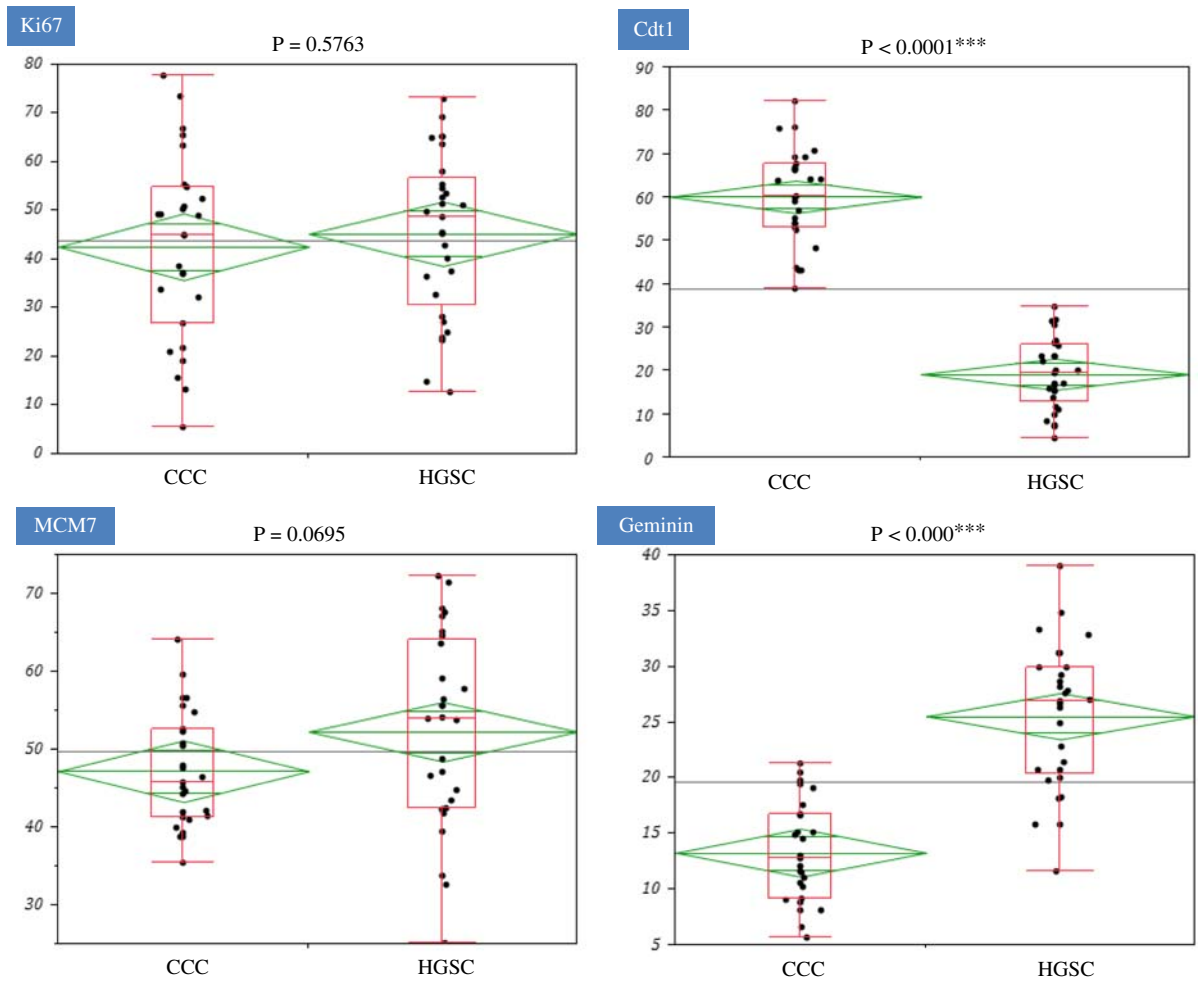


FIG. 4. Comparison of labeling index between clear cell carcinoma (CCC) and high-grade serous carcinoma (HGSC) patients. (1) The expressions of Ki-67 in CCC and HGSC patients were compared. No significant difference in Ki-67 expression between CCC and HGSC was observed. (2) The expressions of MCM7 in CCC and HGSC patients were compared. The expression of MCM7 in HGSC was slightly higher than that in CCC; however, the difference was not significant ($P = 0.07$). (3) The expressions of Cdt1 in CCC and HGSC patients were compared. The expression of Cdt1 in CCC was significantly higher than that in HGSC ($P < 0.0001$). (4) The expressions of geminin in CCC and HGSC patients were compared. The labeling index of geminin in HGSC was significantly higher than that in CCC ($P < 0.0001$).

mitosis by inhibiting the replication factor Cdt1. Degradation of geminin in anaphase allows Cdt1 to promote the binding of MCM proteins, and hence, DNA replication (38). The expression of Cdt1 during cell cycle is high in G1 phase and inhibited by geminin in the S phase of the cell cycle. We focused on the relationship between the expression of Cdt1 and geminin to reveal the difference in the results of immunostaining analysis of cell cycle–related proteins in CCC and HGSC. The present study demonstrated that the expressions of Cdt1 and geminin in CCC were high and low, respectively, than those in HGSC. These results suggest that CCC cancer cells is more abundant during the G1 phase than the other phases of the cell cycle. The present results are consistent with those of the previous studies conducted using cell lines (36). As we did not perform an analysis for G0 phase in the present study, it is difficult to conclude whether G1 phase is the reason for chemotherapy resistance in CCC. However, based on the present study, we concluded that the population of cancer cells of CCC in G1 phase was higher than that of HGSC.

An effective technique of immunostaining analysis and standardization of the count of positive cells for Ki-67 are important factors for successful management of breast cancer. It has been speculated that the standardization of Cdt1 count in ovarian cancer is critical to the application of Cdt1 in clinical management. In the present study, we used the same methodology that was applied in a Japanese validation

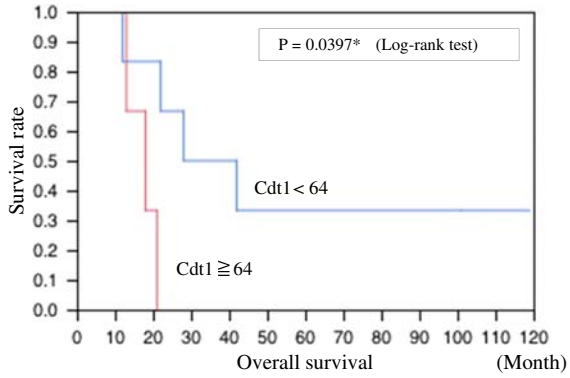


FIG. 5. Cumulative proportion surviving curve (Kaplan-Meier) of high expression of Cdt1 and low expression of Cdt1 in clear cell carcinoma (CCC) patients in the advanced stages (≥ stage 2). Overall survival was compared for CCC patients with high expression of Cdt1 (> 64) and with normal expression of Cdt1 (< 64) in the advanced stages (≥ stage 2). There was a significant difference in overall survival of CCC patients with Cdt1 expression ($P = 0.04$).

ring study, which is the highest intraclass correlation coefficient immunohistochemical analysis of the Ki-67 LI (15). As the Ki-67 LI measured by 6 pathologists without method standardization was in fair-to-good agreement with a study on breast cancer (15), we believe that it is necessary to improve the manual of the system of Cdt1 LI in order to minimize an intraclass correlation coefficient.

The OS of CCC patients in the high positive rate group of Cdt1 (> 64%) was significantly worse than that of CCC patients in the low positive rate group of Cdt1 ($P = 0.004$). These results demonstrated that CCC patients in whom cancer cells mainly existed in G1 phase had poor prognosis. These results also suggested that Cdt1 could be a potential biomarker for predicting the prognosis of CCC. The present study demonstrated that the cell cycle of CCC was different from that of HGSC. It has been speculated that cell cycle–specific chemotherapy is a potential drug for CCC. This possibility should be analyzed using CCC cell lines. Further investigation is needed to propose a new method of CCC treatment.

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