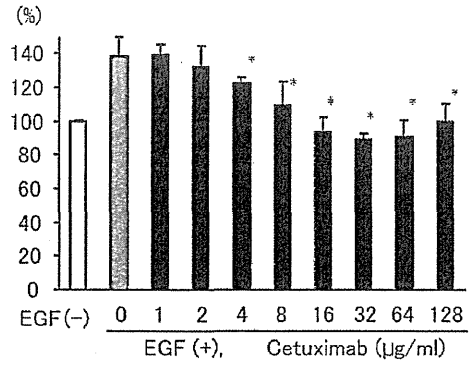

Figure 4. Analysis of the inhibitory effect of cetuximab on *in vitro* cell growth of OMC-1 cells. Cetuximab inhibited *in vitro* cell growth in a concentration-dependent manner. *P<0.01. The results are expressed as mean ± SD.

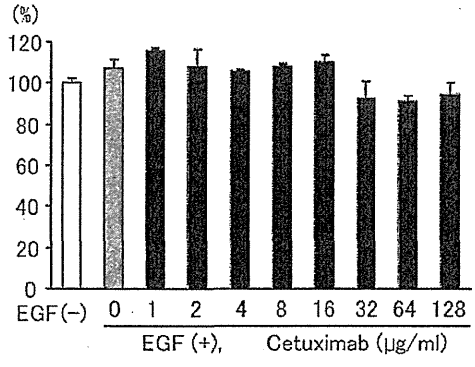

Figure 5. Analysis of the inhibitory effect of cetuximab on *in vitro* cell growth of MCAS cells. Cetuximab did not influence the *in vitro* cell growth of MCAS cells. The results are expressed as mean ± SD.

Effects of cetuximab in vivo. The RMUG-L or MCAS lines of concentrations of $5x10^6$ cells were inoculated subcutaneously into the back of each mouse to induce tumor growth. On the day after inoculation with the tumor cells, cetuximab was intraperitoneally administered 2 times per week at a dose of 1 mg/body until 2 weeks after inoculation. PBS was administered to the control group. The tumor volume [(long diameter) x (short diameter)^2 x 1/2] was measured twice a week to obtain a tumor growth curve.

Statistical analysis. The test of significance between the 2 groups was performed using Student's t-test. A P-value <0.05 was considered significant.

Results

EGFR expression. As shown in Fig. 1, EGFR expression was detected by western blotting at the position corresponding to a molecular weight of 170 kDa. EGFR was detected in all tested cell lines except for the MN-1 line.

KRAS gene mutations. No mutations at codon 12 in exon 2 of the *KRAS* gene were detected in the RMUG-L, RMUG-S, or OMC-1 cell lines. A single point mutation, GGT (Gly) to GAT (Asp), was observed only in the MCAS line at codon 12 in exon 2 of the *KRAS* gene (Fig. 2). The sequence of the MN-1 line was not confirmed because the PCR did not work.

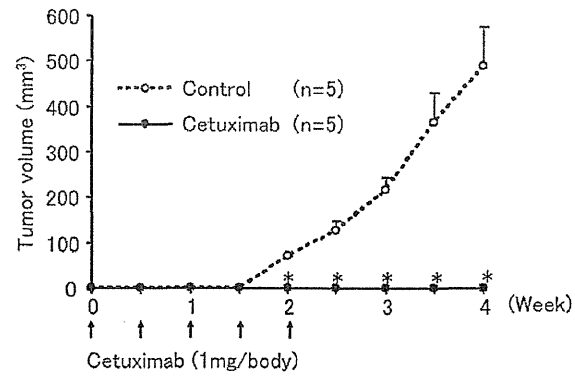

Figure 6. Analysis of the inhibitory effect of cetuximab on *in vivo* tumor growth of RMUG-L cells. The tumor growth was completely abolished at 4 weeks after inoculation in the cetuximab-administered group in comparison with the control group. *P<0.05. The results are expressed as mean ± SD.

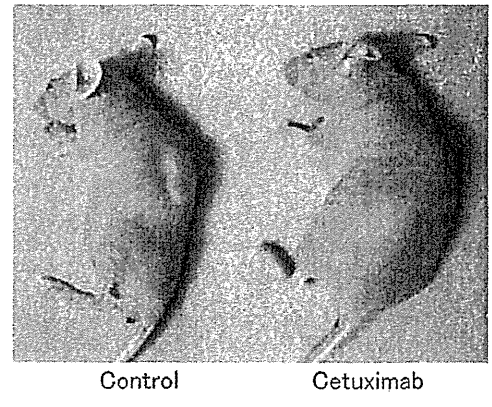

Figure 7. Subcutaneous tumors of RMUG-L cells at 4 weeks after inoculation. A clearly identifiable tumor was formed on the back of the control mouse, while in contrast, no tumor was found on the back of the cetuximab-treated mouse.

Effects of cetuximab on in vitro cell growth. As shown in Figs. 3 and 4, cetuximab inhibited the *in vitro* cell growth of both RMUG-L and OMC-1 cells in a concentration-dependent manner. On the other hand, cetuximab did not influence *in vitro* cell growth of MCAS cells (Fig. 5). Therefore, cetuximab inhibited growth of those MAC cells that lacked a KRAS gene mutation, while cetuximab did not affect growth of MAC cells with a *KRAS* gene mutation.

Effects of cetuximab in vivo tumor growth. As shown in Figs. 6 and 7, RMUG-L tumor growth was completely abolished at 4 weeks after inoculation in the cetuximab-treated group in comparison with the control group. On the other hand, MCAS tumor growth was only partially reduced in the cetuximab-treated group in comparison with the control group (Fig. 8). Therefore, cetuximab completely inhibited MAC tumor growth in those cell lines that lacked a KRAS gene mutation and only partially reduced the MAC tumor growth in those lines with a mutation in the *KRAS* gene.

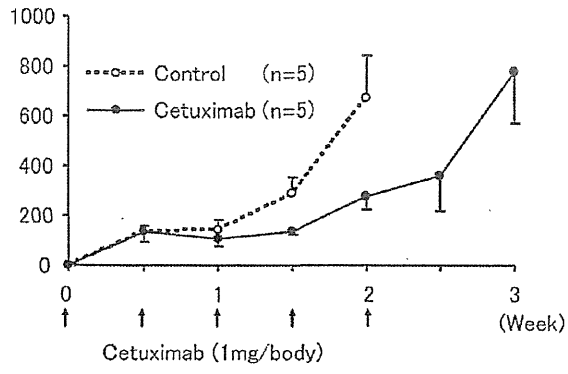

Figure 8. Analysis of the inhibitory effect of cetuximab on *in vivo* tumor growth of MCAS. The tumor growth of MCAS was partially reduced in the cetuximab-treated group in comparison with the control group.

Discussion

In this basic study, we explored the possibility of targeted molecular therapy using cetuximab for MAC in order to develop a new treatment for this disease. First, we investigated the expression of EGFR in 5 MAC cell lines and observed that all of them, except the MN-1 line, expressed EGFR. Next, we screened each cell line for *KRAS* gene mutations and found that only the MCAS line carried a point mutation at codon 12. Finally, we examined the effect of cetuximab on MAC. We observed that cetuximab inhibited the growth of those MAC cell lines that lacked a *KRAS* gene mutation, and by contrast, we showed that cetuximab could not inhibit the growth of MAC cells that carried a mutation in the *KRAS* gene.

MAC is the third most common type of EOC, comprising 10-14% of EOC (5,6). MAC appears to have a distinctly different clinical progression from that of other types of EOC (5,6). Several studies show that MAC is often diagnosed at an early stage, and therefore, it presents a relatively good prognosis (5,6). However, advanced MAC has a poorer prognosis than other histopathologic subgroups (6). MAC's low response (26-42%) to conventional platinum-based chemotherapy is associated with a poor prognosis because chemosensitivity is one of the main prognostic factors for patients with advanced EOC (7-10). Although MAC is known to be resistant to platinum/taxane combination chemotherapy (10), patients with MAC are usually treated with this first-line chemotherapy regimen. A novel treatment strategy for advanced MAC is urgently needed. The histopathology of MAC is similar to that of colorectal cancer. Further, it has been reported that the serum tumor marker and molecular marker expression pattern of MAC differs from those of SAC, and is more similar to colorectal cancer (11). These results suggest that therapeutic agents effective in treating colorectal cancer may also be effective in treating MAC.

Cetuximab is an anti-EGFR monoclonal antibody that binds to EGFR to inhibit its activity, and it is used as a targeted molecular therapeutic agent against specific molecules involved in tumor growth. It is a human-mouse chimeric antibody of the IgG1 subclass and is clinically administered as an intravenous infusion. Recently, cetuximab has been widely used in the medical treatment of colorectal cancer (26). It has been reported that EGFR is expressed in 35-70% of ovarian cancer (13). While there are few studies that have examined MAC, Alshenawy reported that EGFR is expressed in 10 of 21 cases of MAC (16). In the present study, we examined the effect of cetuximab on MAC and found that cetuximab inhibited MAC cell growth *in vitro* and MAC tumor growth *in vivo*. These results suggest the possibility of targeted molecular therapy using cetuximab for MAC.

KRAS, a small G-protein downstream of EGFR and an essential component of the EGFR signaling cascade, can acquire activating mutations in exon 2, thus isolating the pathway from the effect of EGFR (27) and rendering EGFR inhibitors ineffective (28-30). Karapetis *et al* reported that the mutation status of the KRAS gene is associated with overall survival among patients with advanced colorectal cancer who were treated with cetuximab after previous chemotherapy had failed. Compared to only supportive care, treatment with cetuximab was associated with almost a doubling of the median overall and progression-free survival rates among patients with wild-type KRAS tumors. However, there was no significant survival benefit from cetuximab among patients with tumors that had KRAS mutations (26). Mutations in exon 2 of the KRAS gene are observed in ~50% of MAC (31,32). In the present study, cetuximab was unable to inhibit growth of a cell line that had a mutation in exon 2 of the *KRAS* gene. Although this was observed in just 1 cell line, these results suggest that patients with MAC bearing mutated KRAS would not benefit from cetuximab, similar to patients with colorectal cancer. Additionally, in the present study, we observed that cetuximab partially inhibited *in vivo* tumor growth of the cell line that carried the mutation. It has been reported that cetuximab has the potential to inhibit angiogenesis in malignant tumors (33,34) and the potential to enhance a host's tumor immunity (35). Our results described above might reflect these actions.

Thus far, there have been no published studies of targeted molecular therapy for MAC. Targeted molecular therapy for MAC with cetuximab, which we propose here, could be very advantageous for patients with advanced MAC, which is resistant to conventional chemotherapy.

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Fertility-sparing treatment using medroxyprogesterone acetate for endometrial carcinoma

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Abstract. The purpose of this study was to present the results of fertility-sparing treatment using medroxyprogesterone acetate (MPA) for endometrial carcinoma (EC), and to clarify patient characteristics by investigating patient background factors. A total of 59 patients with EC, who received MPA as fertility-sparing therapy at two institutions over a 21-year period between 1987 and 2008, were studied retrospectively. Patients were administered oral MPA at 400-600 mg/day for 16-24 weeks as long as they responded. Endometrial tissue was assessed twice, at 8-12 weeks (during treatment) and shortly after treatment. The overall complete response (CR) rate was 71%. A total of 22 (52%) of 42 responders later developed relapse. A total of 19 cases became pregnant, and 25 infants were born. Eighty percent of recurrences occurred within 2 years. For stages Ia and Ib-IIa (FIGO, 1988), initial CR rates were 80.0 and 42.9%, respectively ($p<0.01$), demonstrating a significant difference. Total hysterectomy was performed for 26 patients (44%) due to recurrence or failure to respond to the initial treatment. Among these 26 patients, postoperative stages were more advanced in 10 patients (38%). The grade advanced (became more poorly differentiated) postoperatively in 2 patients (8%). Premenopausal females with EC can be treated successfully with MPA, however patients should be informed of the risks and limitations of this conservative treatment.

Introduction

In Western countries, endometrial carcinoma (EC) is the most common type of malignant tumor in the field of gynecology (1,2). A state of persistent high estrogen, as observed with ovulation disorders and obesity, is considered a risk factor (3-5). The standard treatments for EC are total hysterectomy and bilateral adnexectomy.

EC is a disease that frequently affects perimenopausal females, however approximately 10% of affected females are ≤40 years old, and the incidence in young females has recently been on the increase (6). As in Western countries, the number of ≤40-year-old females with EC in Japan is on the rise (7). Thus, the number of patients who select to undergo fertility-sparing treatment is growing.

Studies at various institutions have reported the results of fertility-sparing treatment for EC using progesterone preparations (8-17). However, as the number of cases have been low, the efficacy of such treatment has yet to be clarified. Furthermore, few studies have closely described the clinical background of young females with early-stage EC wishing to undergo fertility-sparing treatment.

The present study investigated the effects of treatment, prognosis, pregnancy status and other factors, in order to demonstrate the therapeutic outcomes of current fertility-sparing treatment. Furthermore, patient background factors were investigated to elucidate the characteristics of the patient group.

Patients and methods

Patients. A total of 59 patients with EC who underwent fertility-sparing treatment, at either the Jichi Medical University Hospital or the Kitasato University Hospital over the 21-year period from 1987 to 2007, were retrospectively investigated. Atypical endometrial hyperplasia was excluded in this study. Selection criteria of the treatment were: i) highly differentiated (G1) endometrioid adenocarcinoma; ii) no appearance of myometrial invasion (stage Ia: FIGO, 1988) on magnetic resonance imaging; iii) unmarried or strong desire to have a child; iv) no current or past history of thrombotic disease. As a general rule, blood clotting and fibrinolysis were normal. However, when patients strongly desired, fertility-sparing treatment was performed on patients with stage Ib (in which myometrial invasion may be suspected) or IIa (in which cervical mucosa invasion may be suspected). In all patients, treatment was administered only after written informed

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Key words: endometrial carcinoma, fertility-sparing treatment, medroxyprogesterone acetate

Table I. Patient characteristics.

Patient characteristics, n=59	
Median age, years (range)	31 (21-42)
Mean BMI, kg/m^2 (range)	23.3 (15-38)
Clinical stage (cases)	
Ia	44 (75%)
Ib-IIa	15 (25%)
Risk factor (cases)	
Irregular periods	37 (63%)
Nulligravida	58 (98%)
PCO syndrome	4 (7%)
Detectability (cases)	
Metrorrhagia	35 (59%)
Infertility examination	16 (27%)
Abnormal menses[a]	6 (10%)

[a]Hypermenorrhea/dysmenorrhea/amenorrhea. PCO, polycystic ovary syndrome.

consent was obtained. Pathological specimens were examined by experienced pathologists in each institute.

Treatments. In the fertility-sparing treatment, a dose of 400-600 mg/day of medroxyprogesterone acetate (MPA) was administered orally, and the entire surface was curetted between 8 and 12 weeks to confirm the therapeutic effects and absence of disease progression. Following administration of the drug for 16-24 weeks, the endometrium was again curetted, and therapeutic effects were assessed. At this stage, if the lesion persisted, total hysterectomy was selected as a general rule; if the lesion had disappeared, the patient was monitored and allowed to become pregnant. Even when the lesion persisted, MPA was continued if the patient strongly preferred to preserve fertility. In such patients, pathological tests were conducted every 4-8 weeks, and the duration of therapy was determined individually.

Results

Patient characteristics. A total of 59 patients, including 44 patients with stage Ia (75%) and 15 with stage Ib-IIa (25%) were included in the present study (Table I). The median age was 31 years, and the mean body mass index was 23.3 kg/m^2. The proportions of females with menstrual irregularity, nulliparity and polycystic ovary syndrome (PCO), which are considered risk factors for EC, were 63, 98 and 7%, respectively. The cause of detection was metrorrhagia in 35 patients (59%), and incidental discovery of the lesion during screening or treatment for infertility in 16 patients (27%).

MPA administration positively affects EC patients despite recurrence. In the present study, the mean duration of MPA administration until complete response (CR) was 24.9 weeks (range, 13-70). Fig. 1 shows the therapeutic results. The median duration of follow-up was 66 months (range, 11-251). The response to the initial treatment was CR in 42 patients (71%), and either partial response (PR) or no change (NC) in 17 patients (29%). In 16 of the 17 patients without CR, total hysterectomy was performed and fertility could not be preserved. One patient

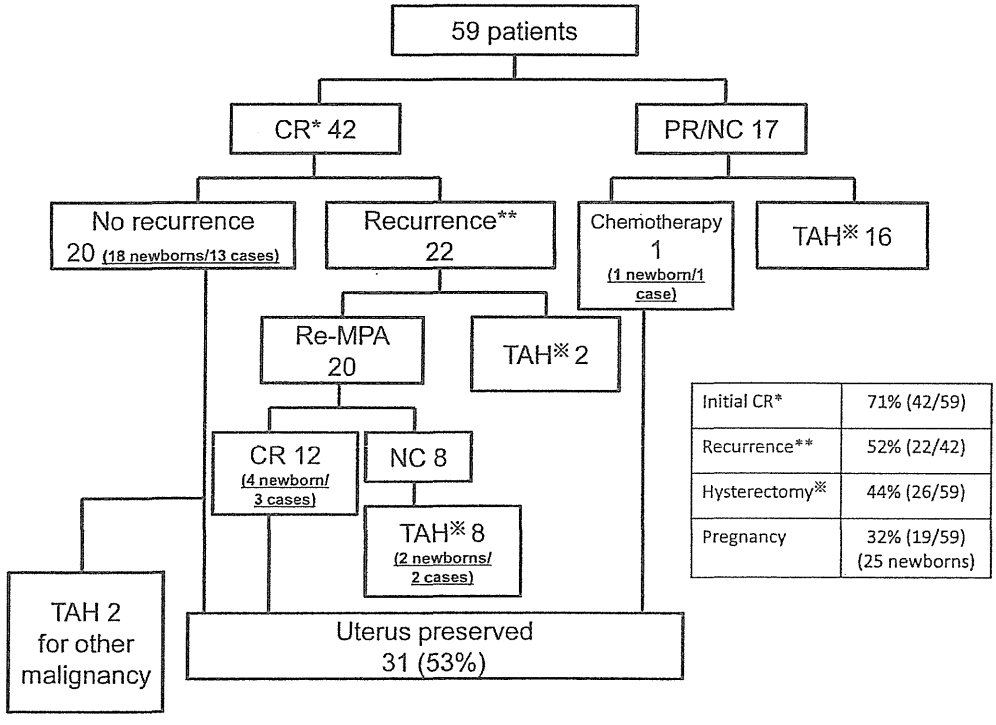
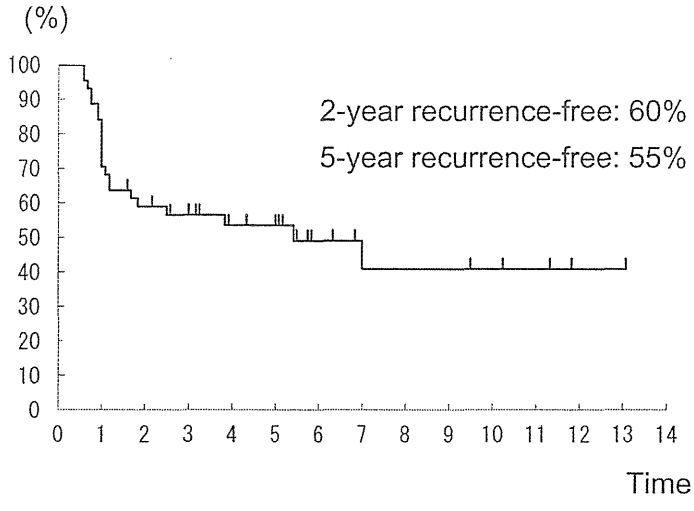


Figure 1. Therapeutic results of fertility-sparing treatment using MPA. MPA, medroxyprogesterone acetate; CR, complete response; PR, partial response; NC, no change; TAH, total abdominal hysterectomy.



Timing of recurrence (years)	n
0-1	13
1-2	4
2-3	1
3-4	1
4-5	0
5-6	1
6-7	0
7-	1

Figure 2. Recurrence-free survival curve and timing of recurrence (Kaplan-Meier analysis).

refused surgery and underwent cytotoxic chemotherapy; subsequently, the cancer went into remission. This patient gave birth to a child and is currently alive and disease-free. Of the 16 patients who underwent surgery, 15 are currently alive and disease-free, but cancer recurred in one patient, who succumbed to recurrence 126 months after initial treatment.

A survey of the 42 initial CR patients revealed that 20 patients (48%) remain alive and are disease-free, with a median recurrence-free survival of 62 months (range, 19-157). Of these 20 patients, 13 patients have given birth to a total of 18 children. However, 2 patients subsequently underwent total hysterectomy; one for new ovarian cancer and the other for new tubal cancer.

Recurrence was observed in 22 patients (52%), with a median onset of recurrence of 12 months (range, 7-84). In 20 of these 22 patients, MPA was again administered, and remission was achieved in 12 patients without recurrence. A total of 3 of these patients have given birth to a total of 4 children. In 8 patients, CR could not be achieved despite additional MPA administration, and total hysterectomy was performed. However, 2 of these 8 patients had each given birth to a child prior to recurrence. Of the 22 patients with recurrence, 2 patients stopped visiting the hospital and have not been followed up.

Fig. 2 shows the timing of recurrence following remission and the recurrence-free survival curve. In 18 (82%) of the 22 patients who had recurrence, cancer recurred within 2 years, and the longest time to recurrence was 7 years and 1 month.

The initial CR rate and the rate of recurrence following CR in relation to the clinical stage. For patients with stage Ia, the CR rate was significantly higher than that for the other stages (80.0 vs. 42.9%, respectively; $p<0.01$). The rate of recurrence was lower for stage Ia patients than recurrence for other stages (47.2 vs. 83.3%, respectively; $p=0.11$), however there was no statistically significant difference (Fig. 3).

Following exclusion of the 2 patients who underwent total hysterectomy for other types of cancer, the 26 patients who underwent total hysterectomy for the primary disease were examined. Clinical and pathological stages matched in

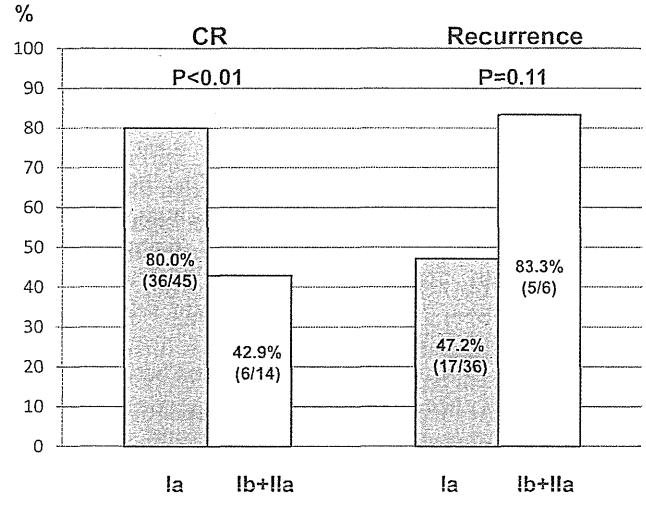


Figure 3. Initial CR rate and rate of recurrence following CR in relation to clinical stage. CR, complete response.

14 patients (54%), however, pathological stages were more advanced in the remaining 10 patients (38%). In 2 patients (8%), the grade as ascertained by biopsy prior to MPA therapy differed from the postoperative grade; grade G1 prior to MPA therapy, but a final postoperative diagnosis of G2 and G3 in each patient, respectively.

As mentioned earlier, double cancer was confirmed in 2 cases (ovarian and tubal cancer), with an overall frequency of 3.3%. In the 2 cases, residual endometrioid adenocarcinoma was not observed in the excised uterus.

Other than mild body weight increases, no adverse reactions were observed with the present treatment. No venous thrombosis was recorded in the patients.

Discussion

The therapeutic results and prognosis of fertility-sparing treatment using MPA for EC were reviewed retrospectively. Table II summarizes findings from past studies. While differences

Table II. Summary of studies on patients treated with progestins.

Author/Year (Refs.)	Number of cases	Complete response (%)	Recurrence (%)
Kim *et al*, 1997 (9)	7	4 (57)	2 (50)
Randall *et al*, 1997 (10)	12	9 (75)	0
Imai *et al*, 2001 (11)	15	8 (53)	3 (37.5)
Kaku *et al*, 2001 (12)	12	9 (75)	2 (22)
Wang *et al*, 2002 (13)	9	8 (89)	4 (50)
Niwa *et al*, 2004 (14)	12	12 (100)	8 (67)
Ushijima *et al*, 2007 (15)	39	26 (67)	14 (54)
Yamazawa *et al*, 2007 (16)	9	7 (78)	2 (29)
Hahns *et al*, 2009 (17)	35	22 (63)	9 (41)
Present study	59	42 (71)	22 (52)
Total	209	147 (70)	66 (45)

exist among institutions in terms of response rates (range, 53-92%) and recurrence rates (range, 11-53%), the present findings were comparable. Our response rate exceeded 70%, and 19 of the patients (32% of enrolled patients) gave birth to a total of 25 children. This suggests that the present treatment is sufficient for the preservation of fertility.

However, patients and doctors require sufficient understanding prior to the initiation of treatment that the recurrence rate for this fertility-preserving treatment is high. As surgery is the standard therapy for EC, the present treatment should be performed only after obtaining written informed consent. As for the preoperative criteria for patient selection, the present study found differences in the rates of initial remission and the rate of recurrence after remission between stages Ia and more advanced stages, and therapeutic results for stages Ia were markedly improved. These results suggest that fertility-sparing treatment for EC should be limited to patients with stages up to Ia.

Our data suggest that for the first 2 years after treatment, patients should be followed up relatively frequently, approximately every 1-3 months, to check for recurrence. As cancer recurred after a long period of remission in certain cases, patients should be followed up for longer than after the first 2 years. Cases have been documented in which EC recurred after more than 10 years (18) or after childbirth (19). In the present study, EC recurred 5 years and 5 months after treatment in one patient (3 years and 10 months after childbirth). Thus, the possibility of recurrence is likely even after several years of remission, as well as after childbirth. These findings suggest that total hysterectomy can be performed as a preventive measure in females in remission who have given birth and do not want to have more children.

An investigation of patients who underwent surgery for recurrence revealed that preoperative clinical stages differed from actual pathological stages in certain cases. Disease stages may have advanced during therapy or preoperative investigations might have missed myometrial invasion. Several studies have revealed the limitations of preoperative staging of EC (8,20). These limitations should be clearly explained to patients prior to therapy, and if a patient does not respond to therapy, the possibility that the stage is more advanced than Ia should be considered. In certain cases, the grade advanced from G1 to G2 or G3 postoperatively. Ota *et al* (21) documented grade progression from G1 to G2 in patients, who were unresponsive to MPA and underwent total hysterectomy. Thigpen *et al* (22) reported that higher grade is associated with lower MPA response rate. As MPA is a long-term treatment, periodic pathological tests are required during follow-up. If grades advance, termination of pharmacotherapy requires consideration versus continuing MPA. As abovementioned, the only patient who succumbed to disease following the present treatment began therapy at stage Ib of G1 endometrioid adenocarcinoma. Surgery was performed due to lack of response to MPA, but by the time of surgery the tumor had advanced to G3 endometrioid adenocarcinoma and had metastasized to the pelvic lymph nodes (pT1cN1M0). Kothari *et al* (23) also reported a patient in whom cancer recurred after fertility-sparing treatment, categorized as stage IV at the time of surgery. Patients need to consider that the present treatment is strictly optional, and that cancer stage can advance during or after MPA.

EC patients who are ≤40 years old are susceptible to complications of ovarian and peritoneal cancer (24). In the present study, patients developing ovarian or tubal cancer were documented. Throughout regular follow-up, sufficient examination of the adnexa of the uterus is required. Furthermore, when totally excising the uterus by radical surgery, the aforementioned types of cancer should be considered when deciding whether to resect the bilateral adnexa, and informed consent must be obtained.

In general, symptoms, including metrorrhagia and postmenopausal bleeding, are observed in a number of EC patients. However, in the present study, approximately 60% of young EC patients who received fertility-sparing treatment had metrorrhagia. Additionally, in approximately 30% of patients, EC was accidentally identified during visits to a hospital for infertility, even though the patient had been asymptomatic during fertility tests. We have already reported that fertility tests detected EC in young females at a higher rate than those for EC in Japanese patients (25). In the group of patients who received fertility-sparing treatment in the present study, EC was detected by fertility tests in a number of patients, suggesting that fertility tests provide an opportunity for the

early detection of EC. Healthcare professionals who conduct fertility tests should remain aware that they are dealing with patients at high risk of EC.

By retrospectively investigating fertility-sparing treatment using MPA for EC, the results and characteristics of patient backgrounds were established. The response rate was high, and the present treatment was considered acceptable for the purpose of enabling patients to give birth. However, the rate of recurrence is also high, thus results remain less effective when compared to surgery, the standard therapy for EC. In addition, standard treatment methods, including daily dose and administration period, have yet to be established. At present, treatments are administered various doese at different institutions. Thus, more studies are required for a standardized treatment to be established.

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Nedaplatin and irinotecan combination therapy is equally effective and less toxic than cisplatin and irinotecan for patients with primary clear cell adenocarcinoma of the ovary and recurrent ovarian carcinoma

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Abstract. This study retrospectively compared nedaplatin and irinotecan hydrochloride (NDP/CPT) combination therapy with cisplatin and irinotecan hydrochloride therapy (CDDP/CPT) for efficacy and adverse events in the treatment of clear cell adenocarcinoma of the ovary (CCC) and recurrent ovarian carcinoma. A total of 115 patients were included in the present study. NDP/CPT was administered intravenously every 4 weeks (NDP, 60 mg/m^2 on day 1; CPT, 50 mg/m^2 on days 1, 8 and 15). CDDP/CPT was also administered intravenously (CDDP, 60 mg/m^2 on day 1; CPT, 60 mg/m^2 on days 1, 8 and 15). Patients with primary CCC were treated with NDP/CPT in 29 cases and CDDP/CPT in 20 cases. Patients with recurrent ovarian carcinoma were treated with NDP/CPT and CDDP/CPT in 33 cases each. No significant difference was observed in the 5-year overall survival (OS)/progression-free survival (PFS) of patients with primary CCC, with the exception of those patients with stages Ia and Ic(b) who underwent NDP/CPT and CDDP/CPT treatments (OS: 58%, PFS: 40% and OS: 53% and PFS: 47%, respectively). No significant differences were found in the response rates to NDP/CPT and CDDP/CPT in patients with recurrent ovarian carcinoma (27 and 18%, respectively). Similarly, there were no significant differences in the 5-year OS and PFS of patients with recurrent ovarian carcinoma treated with NDP/CPT or CDDP/CPT (OS: 15%, PFS: 3% and OS: 18%, PFS: 6%, respectively). In terms of the hematological toxicity of grade 3 or above and non-hematological toxicity of grade 2 or above in patients treated with NDP/CPT and CDDP/CPT, respectively, neutropenia was 23 and 56%; anemia, 1, and 20%; thrombocytopenia, 0 and 5%; nausea, 20 and 52%; diarrhea, 14 and 25%; and fever, 2 and 11%. Accordingly, NDP/CPT indicated mild toxicity, and was therefore equally effective and less toxic than CDDP/CPT in the treatment of primary CCC and recurrent ovarian carcinoma.

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Key words: ovarian cancer, cisplatin, nedaplatin, irinotecan hydrochloride, clear cell carcinoma, recurrence

Introduction

Clear cell adenocarcinoma of the ovary (CCC) is defined by the World Health Organization as lesions characterized by clear cells growing in solid/tubular or glandular patterns as well as hobnail cells (1). The frequency of CCC in female individuals of Western countries is 8% (2). The frequency is higher in Japan at 15% (3). The standard first-line chemotherapy for ovarian epithelial cancer is paclitaxel-platinum combination chemotherapy (T/platinum), which has a response rate of 78% (4). However, for the treatment of CCC, the response rate is only 22-56% (5,6). As such, CCC patients have a poorer prognosis than patients with serous cystadenocarcinoma of the ovary (3). Alternative regimens for the treatment of CCC using combinations of camptothecin derivates, irinotecan hydrochloride and cisplatin (CDDP/CPT) have been investigated (7-9). CDDP/CPT has also been used as an alternative regimen for the treatment of recurrent ovarian carcinoma (10,11). CDDP/CPT is regarded as one of the common treatments of primary CCC and recurrent ovarian carcinoma.

Nedaplatin (cis-diammine glycolato platinum, NDP) is an analog of cisplatin, that shows lower rates of nephrotoxicity and nausea than CDDP. Cervical carcinoma is frequently treated with nedaplatin and irinotecan hydrochloride combination therapy (NDP/CPT). A previous multi-center dose-escalation study was performed to identify appropriate doses of NDP/CPT for cervical carcinoma patients (12). NDP/CPT was found to have a shorter infusion time and lead to a shorter hospital stay than CDDP/CPT.

NDP has been reported to be effective as a single agent or as combination therapy for the treatment of ovarian carcinoma (13,14), and its basic and clinical efficacy in the treatment of CCC has been demonstrated (15,16). Thus, based on our experience, we applied the postoperative chemotherapy regimen for

primary CCC from CDDP/CPT to NDP/CPT. This treatment is also currently used for cases of recurrent ovarian carcinoma.

In this study, we retrospectively compared the efficacy and toxicity of NDP/CPT to that of CDDP/CPT in the treatment of CCC and recurrent ovarian carcinoma.

Patients and methods

Patient characteristics. In total, 115 patients were included in this study, including patients with primary CCC and recurrent ovarian carcinoma were administered CDDP/CPT or NDP/CPT at the Jichi Medical University Hospital, Japan. Subjects had no other known co-morbidities. Informed consent was obtained from all patients.

Inclusion criteria consisted of the following: i) age, ≤75 years; ii) Eastern Cooperative Oncology Group performance status, 0-2; and iii) white blood cell count, ≥3,000/μl; neutrophil count, ≥1,500/μl; platelet count, ≥10x10^4/μl; hemoglobin level, ≥9.0 g/dl; aspartate aminotransferase and alanine aminotransferase levels, ≤3-fold than the upper limit of the normal value; total bilirubin, ≤2.0 mg/dl; urea nitrogen level, ≤25 mg/dl; serum creatinine level, ≤1.5 mg/dl; and creatinine clearance, ≥50 ml/min.

Table I. Characteristics of patients with primary CCC.

	NDP/CPT (n=29)	CDDP/CPT (n=20)
Age (years)		
Median	54	53.5
Range	31-69	37-67
FIGO stage		
Ia/Ic(b)	14	1
Ic(1)/(2)/(a)	5	7
II	4	3
III	4	6
IV	2	3
Residual tumor size (cm)		
0	27	16
<1	2	2
>1	0	2

CCC, clear cell carcinoma; NDP/CPT, nedaplatin plus irinotecan; CDDP/CPT, cisplatin plus irinotecan.

Methods. The NDP/CPT regimen was administered as follows: on day 1, 50 mg/m^2 of CPT was administered intravenously for 90 min, followed by 60 mg/m^2 of NDP for 60 min. On days 8 and 15, patients were administered CPT using the same procedure. Patients were hydrated with 1,000 ml of electrolyte fluids on day 1 only. This was considered as one course and was repeated every four weeks. The CDDP/CPT regimen was administered as follows: CPT at 60 mg/m^2 was administered intravenously for 90 min, followed by CDDP at 60 mg/m^2 for 180 min. On day 8 and day 15, CPT only was administered using the same procedure. On days 0-4, patients were hydrated with 2,000 ml of electrolyte fluids. This was considered as one course and was repeated every four weeks.

Granulocyte-colony stimulating factor was administered for patients with grade 4 neutropenia or grade 3 neutropenia with infection. Granisetron hydrochloride (3 mg), a 5-HT$_3$ receptor antagonist, was administered intravenously on days 1, 8 and 15 as a prophylactic antiemetic treatment. Loperamide hydrochloride, an anti-diarrheal agent, was orally administered at 1-2 mg/day when required. The subsequent course of chemotherapy was administered when the following conditions were met: white blood cell count, ≥3,000/μl; neutrophil count, ≥1,500/μl; platelet count, ≥10x10^4/μl; and absence of diarrhea.

The response to treatment was evaluated by computed tomography (CT) images every 2 cycles of chemotherapy in patients with measurable disease. Tumor response was evaluated according to World Health Organization criteria (1979). A complete response (CR) was defined as the disappearance of all clinical and radiological evidence of the tumor for at least four weeks. A partial response (PR) was defined as a decrease of ≥50% in the sum of the products of the longest perpendicular diameters of all measurable lesions for at least four weeks. Progressive disease (PD) was defined as an increase of >25% in the sum of the products of the perpendicular diameter of all measurable lesions or the appearance of new lesions. Any other events were considered to indicate no change (NC). Progression-free survival (PFS) was defined as the interval from the date of the first chemotherapy administration until the date of recurrence or tumor progression. Overall survival (OS) was defined as the time from the date of chemotherapy until death or the date of the last follow-up contact. Adverse events were graded according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (NCI-CTAE) version 3.0.

Statistical analysis. The Kaplan-Meier method was used to calculate the distribution of patient survival, and its significance in each group was tested using the log-rank test. The χ^2 test was used for statistical analysis. $P<0.05$ was considered statistically significant.

Results

Primary CCC. In the present study, 29 patients with primary CCC were treated using NDP/CPT, and 20 patients were treated using CDDP/CPT (Table I). The median age was 54 years in the NDP/CPT group and 53.5 years in the CDDP/CPT group. According to the International Federation of Gynecology and Obstetrics staging criteria, there were 14 cases with stage Ia/Ic(b) in the NDP/CPT group and only one such case in the CDDP/CPT group. The reason for this observation may be the change in the indication for postoperative adjuvant chemotherapy during the period involved. During the period of CDDP/CPT use, patients with stage Ic(b) did not undergo chemotherapy, whereas during the period of NDP/CPT use, patients with stage Ic(b) underwent chemotherapy. Patients with stage Ia do not usually receive adjuvant therapy; however, if a residual tumor caused by adhesion is suspected, adjuvant therapy is used. There was no significant difference in the degree of completion of surgery.

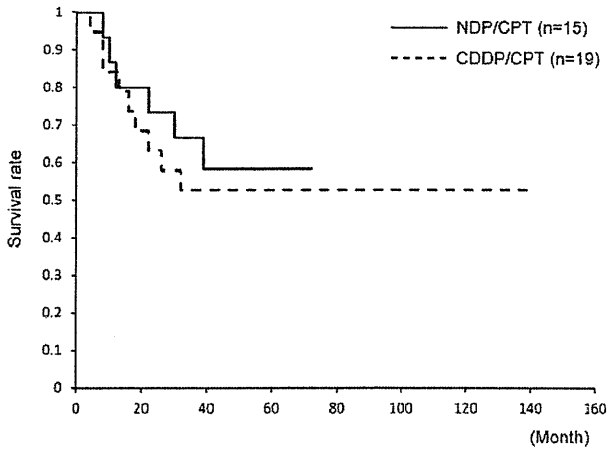

Figure 1. Overall survival (OS) of patients with primary CCC, excluding those with stages Ia and Ic(b). The 5-year OS is 58% in patients treated with NDP/CPT and 53% in those treated with CDDP/CPT. No significant difference was observed.

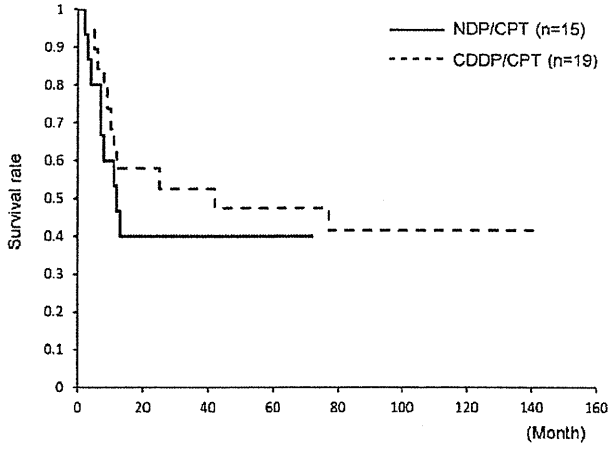

Figure 2. Progression-free survival (PFS) of patients with primary CCC, excluding those with stages Ia and Ic(b). The 5-year PFS is 40% in patients treated with NDP/CPT and 47% in those treated with CDDP/CPT. No significant difference was observed.

Survival was assessed by excluding patients with stage Ia/Ic(b). There was no significant difference in the 5-year OS between the groups; OS was 58% in patients treated with NDP/CPT and 53% in those treated with CDDP/CPT (Fig. 1). Similarly, the 5-year PFS did not significantly differ between the groups; PFS was 40% in patients treated with NDP/CPT and 47% in those treated with CDDP/CPT (Fig. 2).

Recurrent ovarian carcinoma. Sixty-six patients with recurrent ovarian carcinoma were treated with either NDP/CPT or CDDP/CPT (Table II). The median age was 56 years in the NDP/CPT group and 52 years in the CDDP/CPT group. In the two groups, there was a large proportion of patients with stages III-IV, and the most common histological type observed was serous adenocarcinoma. In the two groups, previous treatments mostly consisted of 1 or 2 regimens, and the groups did not show a significant difference. The previous regimen most frequently used in the NDP/CPT group was paclitaxel/carboplatin (TC), whereas it was cisplatin and carboplatin (JP) in the CDDP/CPT group. The NDP/CPT group tended to have a higher 6-month recurrence rate than the CDDP/CPT group; however, this difference was not statistically significant.

The NDP/CPT group had a better response rate than the CDDP/CPT group; however, this difference was not statistically significant (27 and 18%, respectively) (Table III). No significant difference was found in the disease control rate between the two groups (62 and 68%, respectively).

The 5-year OS was not significantly different (15 and 18%, respectively). The median OS was 19 months in the two groups, and it was not significantly different (Fig. 3). The 5-year PFS

Table II. Characteristics of patients with recurrent ovarian carcinoma.

	NDP/CPT (n=33)	CDDP/CPT (n=33)
Age (years)		
Median	56	52
Range	20-72	34-72
FIGO stage		
I	5	5
II	1	3
III	23	15
IV	4	10
Histological subtype		
Serous	19	18
Clear	4	5
Endometrioid	5	3
Mucinous	2	3
Others	3	4
Number of prior chemotherapy regimens		
0	3	2
1	20	19
2	9	10
3	1	2
Prior chemotherapy cycle (duplicated)		
TC	24	13
JP	1	16
Weekly T	8	5
DC	5	0
Other	3	8
Time to recurrence		
<6 months	24	18
>6 months	9	15

NDP/CPT, nedaplatin plus irinotecan; CDDP/CPT, cisplatin plus irinotecan; TC, paclitaxel plus carboplatin; JP, carboplatin plus CDDP; weekly T, weekly paclitaxel; DC, docetaxel plus carboplatin.

Table III. Treatment outcome.

	NDP/CPT (n=33)	CDDP/CPT (n=33)	p
CR	3	3	
PR	4	1	
NC	9	11	
PD	10	7	
NE	7	11	
CR+PR (%)	27% (7/26)	18% (4/22)	ns
CR+PR+NC (%)	62% (16/26)	68% (15/22)	ns

NDP/CPT, nedaplatin plus irinotecan; CDDP/CPT, cisplatin plus irinotecan; CR, complete response; PR, partial response; NC, no change; PD, progressive disease; NE, not evaluable; ns, not significant.

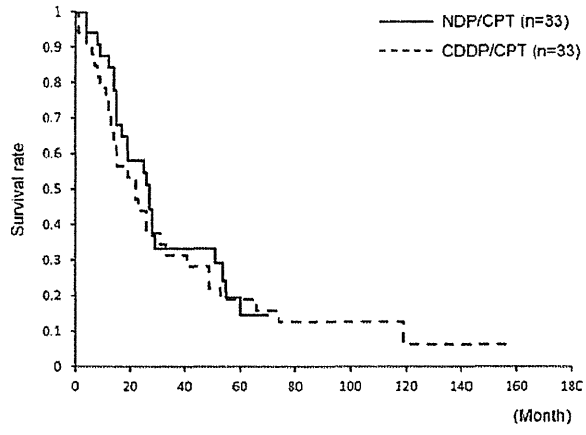

Figure 3. Overall survival (OS) of recurrent ovarian carcinoma. The 5-year OS is 15% in patients treated with NDP/CPT and 18% in patients treated with CDDP/CPT. No significant difference is observed. The median OS in the two groups is 19 months. No significant difference was observed.

was 3% in the NDP/CPT group and 6% in the CDDP/CPT group; however, no significant difference was found between the two groups. The median PFS also did not show any difference (6 and 4 months, respectively, Fig. 4).

Toxicity. Toxicity was assessed among patients with primary CCC and recurrent ovarian carcinoma. Toxicity was compared between the 62 patients treated with NDP/CPT and the 53 patients treated with CDDP/CPT (Table IV). For hematotoxicity of grade 3 or above, neutropenia was observed in 23% of patients treated with NDP/CPT, which was lower than that in patients treated with CDDP/CPT (56%). Anemia and thrombocytopenia were also lower in patients treated with NDP/CPT. With regards to non-hematotoxicity of grade 2 or above, there was a lower rate of nausea in patients treated with NDP/CPT compared to those treated with CDDP/CPT (20 and 52%, respectively). There were also lower rates of diarrhea and fever in the NDP/CPT group.

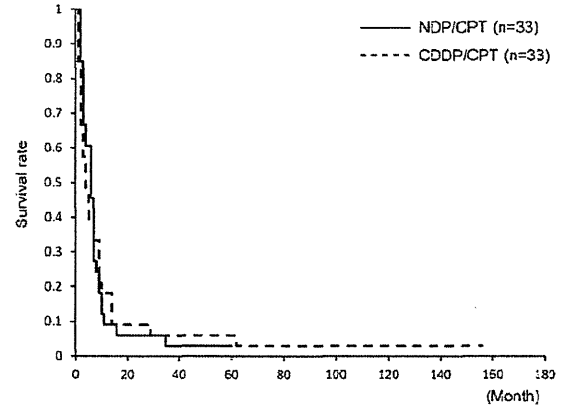

Figure 4. Progression-free survival (PFS) of recurrent ovarian carcinoma. The 5-year PFS is 3% in patients treated with NDP/CPT and 6% in patients treated with CDDP/CPT. No significant difference was observed. The median PFS is 6 months in patients treated with NDP/CPT and 4 months in patients treated with CDDP/CPT. No significant difference was observed.

Discussion

The results of this retrospective analysis demonstrate that NDP/CPT treatment was equally effective yet less toxic than CDDP/CPT in the treatment of primary CCC and recurrent ovarian carcinoma.

NDP is an analog of cisplatin that has the same amine carrier ligands as cisplatin but has a different leaving group, a 5-membered ring structure in which glycolate is bound to the platinum ion as a bidentate ligand. This product is approximately 10 times more water-soluble than cisplatin and, unlike cisplatin, shows limited binding to plasma proteins. As such, it causes less renal, gastrointestinal, and neural toxicity, and there is no need for hydration during its administration (17).

In an *in vitro* study of chemosensitivity, an MTT assay of the ovarian cancer cell strain 65 showed that the inhibition rate of the NDP tumor was 62% and that of CDDP was 55%. For clear cell adenocarcinoma, the inhibition rate of the NDP tumor was 50% and that of CDDP was 37% (15). In a phase II clinical study, patients with ovarian carcinoma who were administered a dose of 100 mg/m^2 of NDP every 4 weeks showed a response rate of 37.7% (23/61). The response rate was 35.7% (15/42) in patients with a previous history of adjuvant therapy, whereas the response rate was 33% in patients with clear cell adenocarcinoma. This study concluded that NDP was equally effective and less toxic than CDDP for the treatment of ovarian carcinoma (13).

CPT is a derivate of camptothecin, and it inhibits topoisomerase I. It may be used either as a single agent or in combination therapy for gastric, colon, lung and cervical carcinomas (18). As a single agent, CPT had a response rate of 20-25% in cases of recurrent or refractory ovarian carcinoma (10).

NDP/CPT has been used as chemotherapy for lung, cervical, and testicular carcinomas (12,19-21). In a phase I study, the Japanese Gynecologic Oncology Group (JGOG) established a recommended dose for patients with cervical carcinoma patients. A phase II study investigating its use as postoperative adjuvant chemotherapy for cervical carcinoma is currently being performed (19). Few available reports

Table IV. Adverse events.

A, Hematological toxicity.

	NDP/CPT					CDDP/CPT					
	n=124 courses				G3/4	n=106 courses				G3/4	
	G1	G2	G3	G4	(%)	G1	G2	G3	G4	(%)	p[a]
Leukopenia	29	51	5	0	4	21	52	24	7	29	<0.01
Neutropenia	16	38	24	5	23	17	28	35	24	56	<0.01
Anemia	26	14	1	0	1	40	37	17	4	20	<0.01
Thrombocytopenia	2	0	0	0	0	21	7	4	1	5	<0.05

B, Non-hematological toxicity.

	NDP/CPT					CDDP/CPT					
	n=124 courses				G2/3/4	n=106 courses				G2/3/4	
	G1	G2	G3	G4	(%)	G1	G2	G3	G4	(%)	p[a]
Nausea	54	24	1	0	20	35	43	12	0	52	<0.01
Diarrhea	29	15	2	0	14	13	16	9	1	25	<0.05
Hepatotoxicity	23	1	1	0	2	6	2	1	0	3	ns
Nephrotoxicity	2	0	0	0	0	1	0	0	0	0	ns
Fervescence	2	2	0	0	2	1	12	0	0	11	<0.01

[a]Statistically significant difference between treatment arms. NDP/CPT, nedaplatin plus irinotecan; CDDP/CPT, cisplatin plus irinotecan; G, grade; ns, not significant.

regarding the use of NDP/CPT for ovarian carcinoma are available; however, one study showed CR to NDP/CPT in patients with CCC metastasis to the common iliac lymph node (16). It was also reported that platinum/CPT therapy, including CDDP/CPT and NDP/CPT, was effective in 31 patients with ovarian carcinoma (14).

The doses of NDP and CPT are determined by body surface area; however, this method may be improved in the future. For instance, Ishibashi *et al* developed Ishibashi's formula to calculate the dose of NDP on the basis of renal function as with carboplatin (CBDCA) (22). The correlation between the predicted area under the curve (AUC) and observed AUC values suggested by Ishibashi's formula was confirmed from a study in which we participated (23). In the future, Ishibashi's formula may be used to determine the appropriate dose of NDP. SN-38, an active metabolite of CPT, is detoxified by glucuronidation with uridine diphosphate gluconosyltransferase (UGT)1A1. The homozygotes and double heterozygotes of UGT1A1*6 and *28 (*6/*6, *28/*28, *6/*28) were significantly associated with severe neutropenia (24). However, the necessity of the dose adjustment of CPT on the basis of the UGT1A1 polymorphism has yet to be determined (25).

Sugiyama *et al* observed that CDDP/CPT treatment had a response rate of 40% in patients with recurrent or refractory ovarian carcinoma (11). Gershenson *et al* reported that platinum-sensitive and non-sensitive ovarian carcinomas had response rates of 75 and 33%, respectively (10). Recently, single-agent chemotherapy has been recommended for cases of platinum-resistant ovarian carcinoma. Platinum-based combination chemotherapy is recommended for platinum-sensitive recurrent ovarian carcinoma (Ovarian Cancer Guideline, http://www.nccn.org/professionals/physician_gls/f_guidelines.asp). Paclitaxel/carboplatin (TC) is often used for platinum-sensitive recurrent ovarian carcinoma. Consequently, the applicability of platinum/CPT is limited for recurrent cases. For patients with platinum-sensitive recurrent ovarian carcinoma that cannot tolerate paclitaxel due to numbness and allergy, NDP/CPT is an alternative chemotherapeutic agent.

Findings of recent studies have suggested that CDDP/CPT has an efficacy similar to or better than T/platinum in patients with primary CCC. In a retrospective study that compared 46 cases of CDDP/CPT and 126 cases of T/platinum with optimal debulking in stages II-IV, the 2-year PFS for CDDP/CPT was 86%, which was higher than that of T/platinum (44%) (7). In another retrospective study of 82 patients treated with TC and 35 patients treated with CDDP/CPT, equal efficacy was observed between the groups (8). In the JGOG's randomized phase II trial, a comparison between 48 cases treated with

CDDP/CPT and 50 cases treated with TC, the treatments were equally tolerated, and there was no significant difference in PFS. Since there were numerous patients in the CDDP/CPT group with large residual tumor cells, a sub-analysis was performed in those with 2-cm residual tumors. In this sub-analysis, the PFS tended to be longer in the CDDP/CPT group, although the difference was not statistically significant (p=0.056) (9).

At present, the JGOG and Gynecologic Cancer Intergroup (GCIG) are participating in an international cooperative randomized phase III trial (GCIG/JGOG3017 ovarian trial) to compare CDDP/CPT and TC as initial first-line chemotherapy for the treatment of CCC (9). If results are obtained that show CDDP/CPT is more suitable than TC for treating patients with CCC, a prospective study should be performed to establish whether a regimen with less toxicity, such as NDP/CPT, is suitable for the treatment of CCC.

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Molecular Cancer Research

Vasohibin-2 Expressed in Human Serous Ovarian Adenocarcinoma Accelerates Tumor Growth by Promoting Angiogenesis

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Abstract

Vasohibin-1 (VASH1) is a VEGF-inducible endothelium-derived angiogenesis inhibitor and VASH2 is its homolog. Our previous analysis revealed that VASH1 is expressed in endothelial cells to terminate angiogenesis, whereas VASH2 is expressed in infiltrating mononuclear cells mobilized from bone marrow to promote angiogenesis in a mouse model of hypoxia-induced subcutaneous angiogenesis. To test the possible involvement of VASH2 in the tumor, we examined human ovarian cancer cells for the presence of VASH2. Immunohistochemical analysis revealed that VASH2 protein was preferentially detected in cancer cells of serous ovarian adenocarcinoma. We then used SKOV-3 and DISS, two representative human serous adenocarcinoma cell lines, and examined the role of *VASH2* in the tumor. The knockdown of VASH2 showed little effect on the proliferation of cancer cells *in vitro* but notably inhibited tumor growth, peritoneal dissemination, and tumor angiogenesis in a murine xenograft model. Next, we stably transfected the human *VASH2* gene into two types of murine tumor cells, EL-4 and MLTC-1, in which endogenous VASH2 was absent. When either EL-4 or MLTC-1 cells were inoculated into *VASH2* (−/−) mice, the VASH2 transfectants formed bigger tumors when compared with the controls, and the tumor microvessel density was significantly increased. VASH2 stimulated the migration of endothelial cells, and its increased expression in cancer cells is related to the decrease of mir-200b. These results indicate that VASH2 expressed in serous ovarian carcinoma cells promoted tumor growth and peritoneal dissemination by promoting angiogenesis. *Mol Cancer Res; 10(9); 1135–46. ©2012 AACR.*

Introduction

Ovarian cancer is the second most common malignant tumor in gynecology and is the leading cause of cancer-related death for women worldwide (1). Cytotoxic therapy with platinum and taxanes is initially effective in many cases of ovarian cancer, but there is a considerable risk of recurrence and resistance to such cytotoxic therapy (2). Thus, it is critical to develop alternative options that target pathways responsible for the progression of ovarian cancer. Angiogenesis is recognized as one of the principal hallmarks of various cancers (3). Indeed, tumor angiogenesis is thought to be a key process that enables ovarian cancer growth as well as dissemination in the peritoneal space, and thus one of the promising options for treating ovarian cancer is considered to be antiangiogenic therapy (4).

Ovarian cancer cells express various angiogenesis stimulators (1). Among them, VEGF plays the most important role, as it stimulates the migration and proliferation of, and tube formation by, endothelial cells. VEGF is the prototype of the VEGF family, and its proangiogenic signals are mainly transmitted via its type 2 receptor (VEGFR2) on endothelial cells (5). High levels of VEGF have been found in ovarian cancers, which is associated with poor survival of patients in both early and advanced stages of the disease (6, 7). In animal models of ovarian cancer, inhibition of VEGF reduces tumor growth and inhibits ascites accumulation (8). It is suggested that VEGF may also promote tumor growth by direct action on VEGF receptors expressed on ovarian cancer cells (9). Angiopoietins and their receptor, TIE2, are another ligand receptor system that regulates angiogenesis. Angiopoietin-1 (Ang-1) is an agonistic ligand of TIE2, and it facilitates pericyte covering of vessels for vascular stabilization, whereas Ang-2 is an antagonistic of ligand of TIE2 and induces detachment of pericytes from vessels for vascular destabilization (10). An increase in the Ang-2 level in cancers may be related to the immature phenotype of tumor vessels.

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Regarding these 2 angiopoietins, the serum Ang-2 level is significantly high in patients with ovarian cancer and is proposed to be a biomarker of malignant potential and poor prognosis in ovarian cancer (11).

The local balance between angiogenesis stimulators and inhibitors determines the occurrence and progress of angiogenesis. We recently isolated vasohibin-1 (VASH1) as a negative feedback regulator of angiogenesis that is induced in endothelial cells by angiogenesis stimulators such as VEGF and fibroblast growth factor 2 (FGF-2; refs. 12, 13). By conducting a database search, we found one gene homologous to VASH1 and named it VASH2 (14). The amino acid sequence of the human VASH2 protein is 52.5% homologous to that of human VASH1, and both VASH1 and VASH2 are highly conserved among species. VASH1 and VASH2 lack the classical signal sequence; but they bind to the small intracellular vasohibin-binding protein (SVBP), and this binding with SVBP facilitates their secretion (15). Because of the similarity between VASH1 and VASH2, we examined their expression and function by the use of hypoxia-induced subcutaneous angiogenesis in mice. Our analysis revealed that VASH1 is mainly expressed in endothelial cells in the termination zone to halt angiogenesis, whereas VASH2 is mainly expressed in mononuclear cells mobilized from the bone marrow in the sprouting front to stimulate angiogenesis (16). Thus, these 2 VASH family members regulate angiogenesis perhaps in a seemingly contradictory manner.

Previously, we investigated the expression of VASH1 under conditions accompanied by conducive to pathologic angiogenesis and showed its presence in endothelial cells of various cancers (17–21), atherosclerotic lesions (22), age-dependent macular degeneration (23), and diabetic retinopathy (24). However, the expression of VASH2 is ill-defined. Here, we conducted immunohistochemical analysis of VASH2 in ovarian cancers and showed for the first time the expression of VASH2 in them. This expression seems to be restricted to serous adenocarcinoma of the ovary. Our subsequent analysis indicated that VASH2 in ovarian cancer cells promoted tumor growth and peritoneal dissemination via the stimulation of angiogenesis.

Materials and Methods

Immunohistochemical analysis of VASH2 in ovarian cancer

This study was approved by the ethics committee of Jichi Medical University Hospital (Tochigi, Japan). Twenty-one patients with epithelial ovarian carcinoma who underwent surgery at Jichi Medical University Hospital between 2007 and 2009 were included in this study. Histologic types were assigned according to the criteria of the World Health Organization (WHO) classification.

Paraffin-embedded blocks of cancer tissue were prepared, and thin sections were cut and placed on glass slides. After deparafinization and hydration, endogenous peroxidase activity was quenched by a 5-minutes incubation in 3% hydrogen peroxide. The sections were then incubated for 1 hour at room temperature with anti-VASH2 mAb (14) at the concentration of 2 μg/mL. The samples were washed with Tris-buffered saline, and the color was developed using the EnVision$^+$System (Dako), according to the manufacturer's instructions. Sections were counterstained with hematoxylin and mounted. A sample from which the primary antibody was omitted served as the negative control.

Cells and cell culture

The human ovarian serous adenocarcinoma cell line SKOV-3 (25) was purchased from American Type Culture Collection (ATCC), and the DISS one was described previously (26). The murine lymphoma cell line EL-4 and murine Lewis lung carcinoma (LLC) were provided from the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan). The murine malignant melanoma cell line B16F1 and murine Leydig tumor cell line MLTC-1 were purchased from ATCC. The murine fibrosarcoma cell line 505-05-01 and the murine ovarian carcinoma cell line OV2944-HM-1 were purchased from RIKEN Cell Bank (Tsukuba, Japan). These cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM; Wako) supplemented with 10% heat-inactivated fetal calf serum (FCS; JRH Biosciences and 100 μg/mL kanamycin (Meiji Seika Kaisha Ltd.). Human umbilical vein endothelial cells (HUVEC) and human microvascular endothelial cells (HMVEC) were obtained from Kurabo Industries, Ltd. and were cultured on type I collagen-coated dishes (IWAKI) in endothelial basal medium EBM-2 (Lonza) supplemented with EGM-2-MV-SingleQuots (Lonza) containing VEGF, FGF-2, insulin-like growth factor-I, EGF, and 5% FBS. MS1, an immortalized cell line with a SV40 large T antigen from mouse pancreatic endothelial cells, was purchased from ATCC and was cultured in αMEM (Wako) supplemented with 10% FCS. Human ovarian epithelial cells (HOEC) were purchased from ScienCell Research Laboratories, and were cultured on poly-L-lysine–coated dishes (IWAKI) in ovarian epithelial cell medium (ScienCell) supplemented with ovarian epithelial cell growth supplement (ScienCell).

All the cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Knockdown of VASH2 by short hairpin RNA

The short hairpin RNA (shRNA) sequence that suppresses VASH2 expression is 5′-CACCAGGTGATCTAGAATT-GCATACGTGTGCTGTCCGTATGTAATTCTGGAT-CGCCTTTTTT. This sequence was inserted into the piGENE hU6 Vector (iGENE) to make the VASH2 shRNA expression vector. Cells were transfected with this vector or control vector by using Effectene transfection reagent. After the transfection, the cells were selected in puromycin (Calbiochem)-containing medium; and the bulk cells were obtained. Next, the bulk cells were seeded at a density of 0.3 cells per well in 96-well plates with 100 μL medium per well, and visible clones were picked and expanded in 24-well plates. These clones were finally transferred to regular cell culture flasks, and the VASH2 knockdown clones were thus established.

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Establishment of VASH2-expressing murine tumor cell clones

Human *VASH2* cDNA was cloned into the pCALL2-pcDNA3.1/Hygro vector at multiple cloning sites (*Xho*I and *Not*I). To improve the activity of transcription, we replaced the cytomegalovirus (CMV) promoter of the pcDNA3.1/Hygro plasmid (Invitrogen) with the chicken *β-actin* promoter derived from pCALL2 (27). Cells were transfected with the *VASH2* expression vector or control vector by using Effectene transfection reagent (QIAGEN) according to the manufacturer's protocol. After the transfection, the cells were selected in hygromycin (Invitrogen)-containing medium, and the clones having high VASH2 expression were established according to the procedure similar to that for the selection of the VASH2 knockdown clones.

Reverse transcriptase PCR

Total RNA was extracted from cell cultures by using an RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. Total tissue RNA was extracted from tumors with ISOGEN (Nippon Gene) according to the manufacturer's instructions. First-strand cDNA was generated by using ReverTra Ace (TOYOBO). The reverse transcriptase PCR (RT-PCR) procedure was carried out in a DNA thermal cycler (Takara). PCR conditions consisted of an initial denaturation step at 95°C for 5 minutes followed by 25 to 35 cycles consisting of 15 seconds at 95°C, 15 seconds at the appropriate annealing temperature, and 30 seconds at 72°C. PCR products were separated on a 1.5% agarose gel and visualized under ultraviolet by ethidium bromide staining. The primer pairs used were as follow: mouse *β-actin* forward, 5′-TCGTGCGTGACATCAAAGAG, and reverse, 5′-TGGACAGTGAGGCCAGGATG (annealing temperature: 58°C); human *β-actin* forward, 5′-ACAATGAGCTGCGTGTGGCT, and reverse, 5′-TCGTGCGTGACATTAAGGAGA (annealing temperature: 58°C); mouse *VASH2* forward, 5′-TGGAGACAGCGAAGGAGATG, and reverse, 5′-GAAGCAACTTGTCCTCAACG (annealing temperature: 56°C); human *VASH2* forward, 5′-AGCTGATGGACAAGCCATTG, and reverse, 5′-CTCTGAATGAAGTGGGCTATC (annealing temperature: 56°C).

Transient transfection with anti-miR or pre-miR

SKOV-3 cells were grown to 60% to 70% confluence and then transfected with 50 nmol/L anti-miR-200b (Ambion), pre-miR-200b (Ambion), or their negative control oligonucleotides (Ambion) by using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. The medium was changed after 12 hours of transfection, and the cells cultured for an additional 48 hours.

Quantitative real-time RT-PCR of VASH2 and miR-200b

Total RNA was extracted from HOECs, ovarian cancer cells, and ovarian cancer tissues using RNeasy Mini Kit. First-strand cDNA was generated using ReverTra Ace for RT-PCR. Quantitative real-time RT-PCR was carried out using the CFX96 real-time PCR detection system (Bio-Rad Laboratories) according to the manufacturer's instructions. PCR conditions consisted of an initial denaturation step at 95°C for 3 minutes, followed by 40 cycles of 10 seconds at 95°C, 10 seconds at 56°C, and 30 seconds at 72°C. Each mRNA level was measured as a fluorescent signal corrected according to the signal for *β-actin*. The primer pairs used were as follows: human VASH2 forward, 5′-TGCACACAGTCAAGAAGGTC-3′, and reverse, 5′-TTCTCACTTGGGTCGGAGAG-3′; human *β-actin* forward, 5′-ACAATGAGCTGCGTGTGGCT-3′, and reverse, 5′-TCTCCTTAATGTCACGCACGA-3′. miR-200b levels were analyzed by the TaqMan real-time PCR method. Ten nanograms of total RNA was reverse transcribed by using a TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. The specific primer for miR200b was designed and produced by Applied Biosystems. Real-time PCR was carried out with the CFX96 real-time PCR detection system. PCR conditions consisted of an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Relative expression levels were calculated by using the comparative C_t method.

Proliferation of tumor cells

Proliferation of tumor cells was measured by conducting the Tetra Color ONE cell proliferation assay (28). Briefly, the cells were seeded at a density of 3×10^3 cells per well in a 96-well plate and incubated at 37°C. After 48 hours, 5 μL of Tetra Color ONE (Seikagaku Co.) was added to each well; and mixture was then incubated for an additional 2 hours. Absorbance at 450 nm was monitored.

Proliferation of endothelial cells

VASH2-expressing EL-4 clone or EL-4 cells transfected with empty vector were cultured at 1×10^6 cells/mL for 24 hours and centrifuged at 1,000 rpm for 5 minutes to obtain the conditioned medium (CM). Next, the cellular components were removed from the CM by using a MILLEX-GP PES 0.22-μm filter (Millipore) and concentrated 10-fold with a VIVASPIN15; MWCO 10,000 (Sartorius Stedim Biotech). MS1 cells were plated in a 96-well plate at 2×10^3 cells per well and cultured in medium to which the CM had been added, and the proliferation was measured by using the Tetra Color ONE.

Migration of endothelial cells

The migratory activity of endothelial cells was measured by use of the modified Boyden chamber method. MS1 cells were preincubated in 1% FCS in α-MEM for 24 hours and then plated at 5×10^5 cells/mL on the upper chamber (insert) of a Boyden chamber (8.0-μm pore size, Corning). The low chamber was filled with CM corrected from *VASH2* gene or mock transfectants as described above. MS1 cells were allowed to migrate for 6 hours, the cells that had migrated across the filter were

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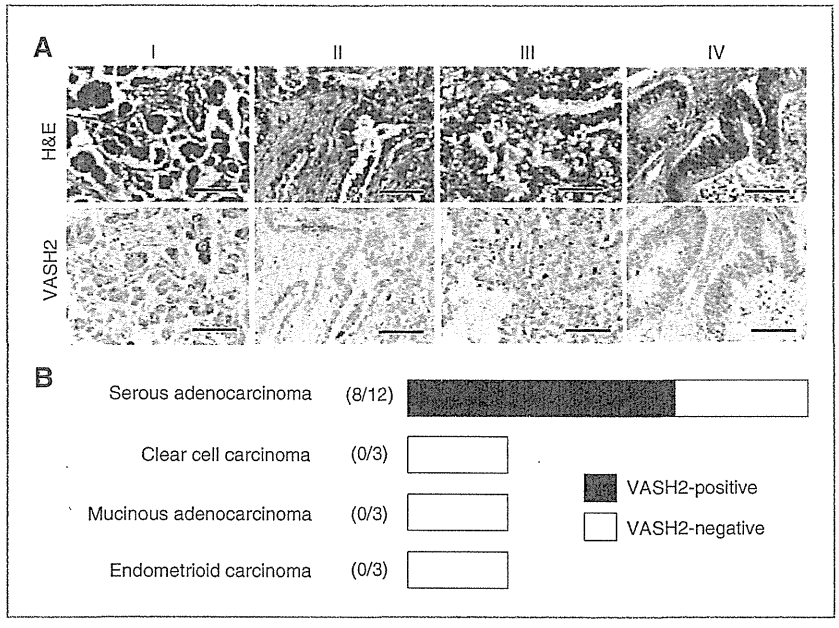

Figure 1. Expression of VASH2 in human serous ovarian adenocarcinoma cells. A, histologic analysis of human ovarian adenocarcinoma was conducted. I and II, serous ovarian adenocarcinoma; III, clear cell carcinoma; and IV, mucinous adenocarcinoma. Top, hematoxylin and eosin (H&E) staining. Bottom, VASH2 immunostaining. Bar, 100 μm. B, eight of 12 cases were positive for VASH2 in serous ovarian adenocarcinoma. Three cases of clear cell carcinoma, 3 cases of mucinous adenocarcinoma, and 3 cases of endometrioid carcinoma were all negative for VASH2.

stained with Difu Quick (Sysmex), and the number of cells that had migrated was counted in 5 fields per insert in a blind manner.

Mouse xenograft models

Female 6- to 8-week-old BALB/c nude mice were obtained from Clea Laboratories. *VASH2*$^{-/-}$ mice on a C57BL/6 background were previously described (16). All of the animal experiments were approved by Tohoku University Center for Gene Research and carried out under the guidelines for animal experimentation of Tohoku University.

Subcutaneous tumor growth

Tumor cells were subcutaneously transplanted into the back of mice at 2×10^6 cells per mouse. Two dimensions of the tumors were measured every 3 days by using a caliper. The tumor volume was calculated by the formula: volume = (short diameter)^2 × (long diameter) × 1/2.

Peritoneal dissemination, ascites accumulation, and survival rate

Tumor cells were intraperitoneally injected into BALB/c nude mice at 2×10^6 cells per mouse. Two or 3 weeks after the injection, the mice were sacrificed. Thereafter, the ascites fluid was collected, and its volume was measured. Peritoneal dissemination was evaluated by counting the number of tumor nodes on the surface of the small intestines and mesentery. The survival of the mice was monitored twice daily. The survival rate was calculated by the Kaplan–Meier method.

Orthotopic inoculation of ovarian cancer cells

Orthotopic tumor cell inoculation was conducted according to the method described by Cordero and colleagues. (29). Briefly, a small incision was made at the dorsomedial position and directly above the ovarian fat pad. The ovarian fat pad was gently pulled out and cancer cells (1×10^5) were inoculated between the brusa and the ovary. Thirty-two days after the inoculation, mice were sacrificed and tumors in the ovary were examined.

Immunohistochemical analysis

For immunohistochemical analysis, tumors were frozen in optimum cutting temperature (OCT) compound (Sakura), cut into 7-μm sections, fixed in methanol for 20 minutes at −20°C, and blocked with 1% bovine serum albumin in PBS for 30 minutes at room temperature. Primary antibody reactions were conducted overnight at 4°C with rat monoclonal antibody against mouse CD31 (Research Diagnostics) at a dilution of 1:500, mouse monoclonal antibody against mouse αSMA (Sigma-Aldrich) at a dilution of 1:200. Secondary antibody reactions were conducted for 1 hour at room temperature with Alexa Fluor 488–conjugated donkey anti-rat IgG, Alexa Fluor 568–conjugated goat anti-rat IgG, Alexa Fluor 555–conjugated goat anti-mouse IgG (Molecular Probes) at a dilution of 1:500. After having been washed

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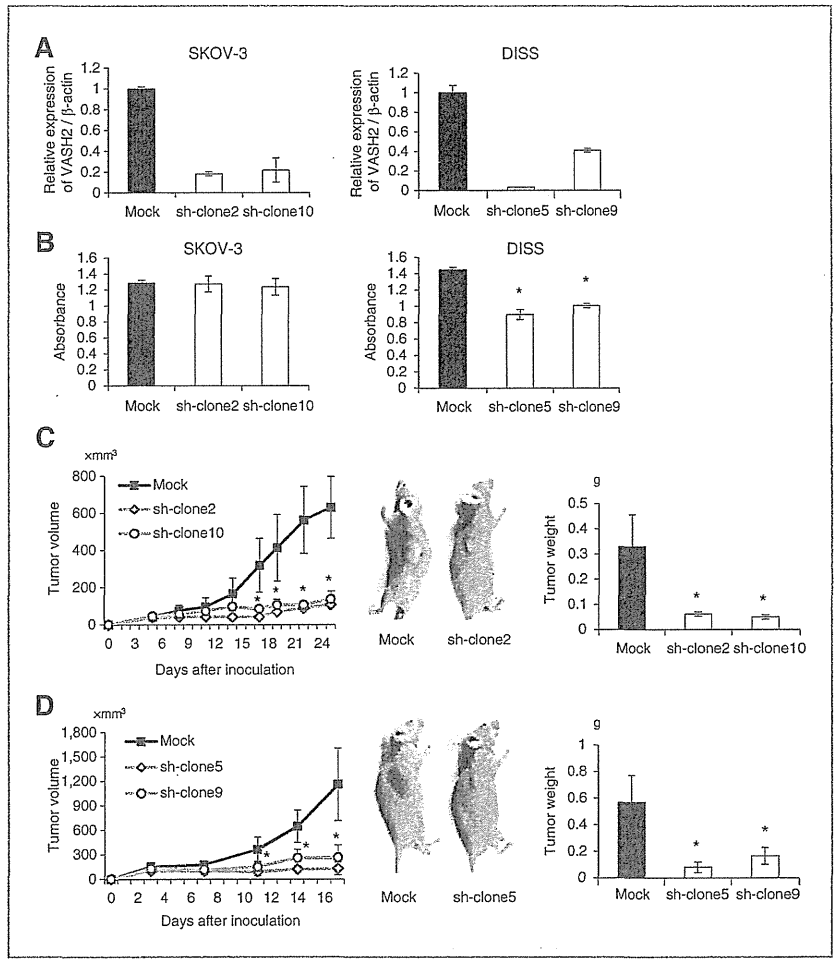

Figure 2. Knockdown of VASH2 inhibited subcutaneous tumor growth. A, two VASH2 knocked-down (sh-VASH2) clones (sh-clone2 and sh-clone10) from SKOV-3, 2 from DISS (sh-clone5 and sh-clone9), and their control mock transfectants were established. Their expression of VASH2 was determined by quantitative RT-PCR. Means and SDs are shown ($N = 3$). B, proliferation of sh-VASH2 clones and of their control mock transfectant was compared under the same cell culture conditions. Means and SDs are shown ($N = 3$). *, $P < 0.01$ versus mock. C, two sh-VASH2 clones (sh-clone2 and sh-clone10) or their control mock established from SKOV-3 cells were inoculated subcutaneously into nude mice, and the serial tumor growth was compared in terms of tumor volume and weight. Means and SDs are shown ($N = 5$). *, $P < 0.01$ versus mock. Twenty-five days after the inoculation, photographs were taken, and the tumor weight was measured. Mean and SDs are shown ($N = 5$). *, $P < 0.01$ versus mock. D, two sh-VASH2 clones (sh-clone5 and sh-clone9) or their control mock established from DISS were inoculated subcutaneously into nude mice and the serial tumor growth was compared as in C. Means and SDs are shown ($N = 4$). *, $P < 0.01$. Seventeen days after the inoculation, photographs were taken, and tumor weight was measured. Mean and SDs are shown ($N = 4$). *, $P < 0.01$ versus mock.

3 times with PBS, the sections were covered with fluorescent mounting medium. All samples were analyzed with a BZ-9000 fluorescence microscope (KEYENCE) with a ×10, ×20, ×40, ×100 objective lens at room temperature. The vascular luminal area was calculated from 5 different fields. Quantitative analyses of vessels and cells were done by using BZ-H1C software (KEYENCE) and ImageJ software (http://rsbweb.nih.gov/ij/).

Quantification of proliferating cancer cells *in vivo*

Deparaffinized and rehydrated tumor tissue sections were incubated overnight at 4°C with anti–proliferating cell nuclear antigen (PCNA) antibody (Santa Cruz Biotechnology) at a dilution of 1:200. The samples were incubated in biotin-conjugated antibody solution and streptavidin followed by staining with 3,3′-diaminobenzidine (DAB). The sections were then counterstained with Mayer hematoxylin, and the percentage of PCNA-positive cells was quantified by using HistoQuest software (NOVEL SCIENCE).

Quantification of apoptotic cancer cells *in vivo*

To evaluate apoptotic cancer cells, we conducted terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining. Deparaffinized and rehydrated tumor tissue sections were immersed in protease solution and were incubated with terminal deoxynucleotidyl transferase to label 3′ terminals of DNA. Then, they were incubated in peroxidase-conjugated antibody solution and were stained with DAB. The sections were counterstained with methyl green, and the percentage of TUNEL-positive cells was quantified by using HistoQuest software (NOVEL SCIENCE).

Statistical analysis

The statistical significance of differences was evaluated by unpaired ANOVAs, and probability values were calculated with the Student t test. Survival rates were analyzed by the generalized Wilcoxon and log-rank tests. $P < 0.05$ was considered statistically significant.

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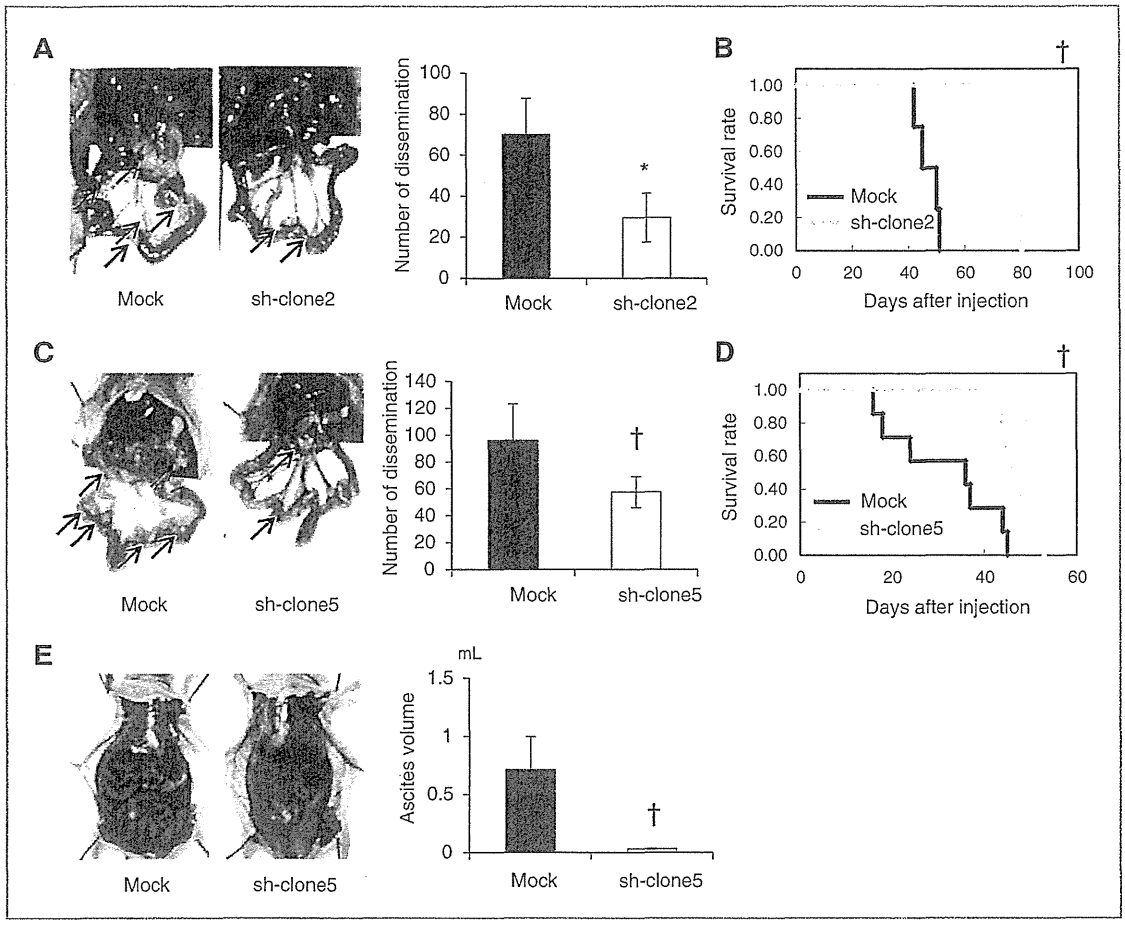

Figure 3. Knockdown of VASH2 inhibited peritoneal dissemination of tumor cells and improved survival rate *in vivo*. A, sh-clone2 or mock transfectant cells established from SKOV-3 were injected into nude mice by the intraperitoneal route. Three weeks after the injection, the mice were sacrificed; and then peritoneal dissemination of the tumor cells was determined. Mean and SDs are shown ($N = 4$). *, $P < 0.01$ versus mock. B, after the injection of sh-clone2 or mock transfectant established from SKOV-3, the survival of the mice was monitored twice daily ($N = 4$). †, $P < 0.05$ versus mock. C, sh-clone5 or mock transfectant cells established from DISS were injected into nude mice intraperitoneally. Two weeks after the injection, the mice were sacrificed; and peritoneal tumors were counted. Mean and SDs are shown ($N = 3$). †, $P < 0.05$ versus mock. D, after the injection of sh-clone5 or mock transfectant cells established from DISS, the survival of the mice was monitored twice daily ($N = 7$). †, $P < 0.05$ versus mock. E, sh-clone5 or mock transfectant cells established from DISS were injected into nude mice intraperitoneally. Two weeks after the injection, the mice were sacrificed, after which the volume of ascites was measured. Mean and SDs are shown ($N = 3$). †, $P < 0.05$ versus mock.

Results

VASH2 is expressed in human serous ovarian carcinoma cells

To test the possible involvement of VASH2 in the tumor, we analyzed human ovarian cancers for its presence. Immunohistochemical analysis of the pathologic sections revealed that VASH2 protein was preferentially detected in serous ovarian adenocarcinoma cells (Fig. 1A). Indeed, cancer cells in 8 of 12 cases of serous ovarian adenocarcinoma (67%) were positive for VASH2, whereas cancer cells in all of the cases of non–serous ovarian adenocarcinoma; that is, 3 cases of clear cell carcinoma, 3 cases of mucinous adenocarcinoma, and 3 cases of endometrioid carcinoma, were negative for it (Fig. 1B).

Knockdown of VASH2 in cancer cells decreases tumor growth via the inhibition of tumor angiogenesis

To verify the function of VASH2 in ovarian cancers, we conducted a loss-of-function experiment by knocking down the expression of VASH2. We used SKOV-3 and DISS for the following experiments, as they are representative ovarian cancer cells that are highly tumorigenic in animals. At least, 7 variant transcripts of human VASH2 are registered in the database (Supplementary Fig. S1). We designed shRNAs to knock down most of the splicing variants and established 2 VASH2 knocked-down (sh-VASH2) clonal cell lines from each of SKOV-3 and DISS (Fig. 2A). The knockdown of VASH2 did not alter the *in vitro* proliferation of SKOV-3 but slightly decreased that