Topoisomerase-I treatment induces an increase in the S-phase cell population with an increase in topoisomerase-II mRNA expression. Thus, topoisomerase-I can modulate topoisomerase-II levels to enhance the effect of topoisomerase-II inhibitors (13,14).

Eder et al. (15) reported the result of the *in vivo* study. They showed that a combination of irinotecan and etoposide showed more than an additive effect by both the tumor excision assay and tumor growth delay assay.

A Phase I study of topotecan and oral etoposide revealed severe myelosuppression but promising efficacy for ovarian cancer (16).

The dose-limiting toxicity of irinotecan is diarrhea, different from that of topotecan (myelosuppression). Then, utilizing etoposide with irinotecan may improve the risk–benefit balance of dual inhibition of topoisomerase. The result of the Phase I study was reported in ASCO 2002 (17).

The recommended dose for further study was oral etoposide: 50 mg/m^2/days 1–21 and intravenous irinotecan: 60 mg/m^2/days 1 and 15, repeated every 4 weeks.

In this Phase I study, four objective responses [two complete responses and two partial responses (PRs)] were achieved among 24 patients, including one PR in clear cell.

Nishio et al. (18) reported the result of feasibility study run by selected hospitals in Tohoku and Kyushu districts in Japan. Response rate, time to progression and overall survival were 44%, 9 months and 17 months, respectively.

This very promising result lead us to conduct a nationwide Phase II study run by Japan Clinical Oncology Group (JCOG).

The protocol review committee of the JCOG approved this protocol in January 2009 and the study was initiated in April 2009. This trial was registered at UMIN-CTR as UMIN000001837 (http://www.umin.ac.jp/ctr/index.htm).

PROTOCOL DIGEST OF THE JCOG0503

Objectives

The aim of this study is to evaluate the safety and efficacy of oral etoposide and intravenous irinotecan for patients with platinum-resistant and taxane-pretreated ovarian, tubal and peritoneal cancer as the test arm regimen in a subsequent Phase III trial.

Study Setting

The study is a multi-institutional open-label two-stage design Phase II trial.

Resources

This study is supported by Grants-in-Aid for Cancer Research (20S-1 and 20S-6) and Health and Labor Sciences Research Grant for Clinical Cancer Research (18–6), from The Ministry of Health, Labor and Welfare of Japan.

Endpoints

The primary endpoint is response rate in all eligible patients. For patients with measurable lesion, response is evaluated according to the RECIST criteria (19). For patients with non-measurable lesion, response is evaluated according to the GCIG CA-125 criteria (20). The secondary endpoints are progression-free survival, overall survival and adverse events. Overall survival is defined as days from registration to death from any cause, and it is censored at the last follow-up day when the patient is alive. Progression-free survival is defined as days from registration to disease progression (either of radiological, CA-125, symptomatic) or death from any cause, and it is censored at the latest day when the patient is alive without any evidence of progression.

Eligibility Criteria

Inclusion Criteria

For inclusion in the study, patients are required to fulfill all of the following criteria:

(i) cytologically or histologically proven ovarian, tubal or peritoneal cancer
(ii) platinum-resistant disease
(iii) taxane-pretreated disease
(iv) age: 20–75 years old
(v) PS (performance status): 0–2
(vi) one of the followings, or both of them:
  (a) patients have measurable lesion
  (b) patients have assessable lesion with elevated CA-125 (more than 70 U/ml)
(vii) no prior treatment with irinotecan, topotecan or etoposide
(viii) no prior radiation to abdomen
(ix) oral intake without parenteral nutrition
(x) both of the followings:
  (a) no drainage to effusion or ascites within 28 days
  (b) no effusion or ascites to be drained at registration
(xi) both of the followings:
  (a) no chemotherapy or surgery within 28 days
  (b) no hormonal or biologic therapy within 14 days
(xii) patients without severe organ dysfunction
(xiii) written informed consent

Exclusion Criteria

Patients are excluded if they meet any of the following criteria:

(i) synchronous or metachronous (within 5 years) malignance other than carcinoma *in situ* or intramucosal cancer
(ii) mental disease or mental symptoms that would affect the participant's decision to participate
(iii) pregnant or lactating
(iv) continuous systemic steroid

(v) active bacterial or fungal infection with fever of 38.5°C or higher
(vi) uncontrollable hypertension
(vii) uncontrollable diabetes requires continuous insulin administration
(viii) history of myocardial infarction or heart failure within 6 months, or current unstable angina
(ix) bowel obstruction

Treatment Methods

Etoposide is orally administered once a day at 50 mg/m^2 from days 1 to 21, and irinotecan is infused at 70 mg/m^2 on days 1 and 15, repeated every 28 days. Protocol treatment is continued up to six cycles unless disease progression, unacceptable toxicity or patient refusal.

Follow-up

Enhanced abdominal computed tomography (CT)/magnetic resonance imaging, chest CT/X-rays and tumor marker (CA-125) are evaluated at least every 8 weeks during the protocol treatment. Adverse events are evaluated at least every 2 weeks during the protocol treatment using CTCAE ver. 3.0.

Study Design and Statistical Analysis

This study is a Phase II trial with two-stage design by Southwest Oncology Group (21) to evaluate this regimen as the test arm for a subsequent Phase III trial.

The sample size was determined as follows by the SWOG design. We assumed that the expected value for the primary endpoint of 35% and the threshold value of 20%. In this situation, the sample size ensuring at least 80% power with one-sided α of 5% is 55. Considering the likelihood of some ineligible patients being enrolled, the total number of patients was set at 60.

Interim Analysis and Monitoring

We plan interim analysis for futility after 30 patients enrolled. In house monitoring will be performed every 6 months by the JCOG Data Center to evaluate the study progress and to improve the study quality.

PARTICIPATING INSTITUTIONS

The participating institutions (from north to south) are as follows: Hokkaido University Hospital, Sapporo Medical University, Iwate Medical University, Tohoku University Hospital, Institute of Clinical Medicine, Tsukuba University, National Defense Medical College, Saitama Cancer Center, Saitama Medical Center, Saitama Medical School, National Cancer Center Hospital, Jikei Kashiwa Hospital, Tokyo Metropolitan and Infectious diseases Center Komagome Hospital, The University of Tokyo Hospital, Jikei University Hospital, Cancer Institute Hospital, Juntendo University School of Medicine, Kitasato University School of Medicine, Niigata Cancer Center Hospital, Shinshu University School of Medicine, Aichi Cancer Center Hospital, Kyoto University Hospital, Osaka city University Hospital, Kinki University School of Medicine, Osaka Medical Center for Cancer and Cardiovascular Disease, Osaka City General Hospital, Sakai Hospital, Kinki University School of Medicine, Hyogo Cancer Center, Tottori University, Kure Medical Center Chugoku Cancer Center, Shikoku Cancer Center, Kyushu Cancer Center, Kurume University School of Medicine, Kyushu University Hospital, Faculty of Medicine Saga University, Kumamoto University Medical School, Kagoshima City Hospital and University of the Ryukyu.

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Conflict of interest statement

None declared.

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A multi-institutional phase II trial of paclitaxel and carboplatin in the treatment of advanced or recurrent cervical cancer

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ABSTRACT

Objective. The aim of this prospective trial was to evaluate the efficacy and safety of the combination of paclitaxel and carboplatin (TC) in patients with metastatic or recurrent cervical cancer.

Methods. This was a multicenter phase II trial of 3 weekly paclitaxel 175 mg/m^2 3-hour iv day 1 followed by carboplatin AUC5 1-hour iv day 1 for maximum of 6 cycles until disease progression or prohibitive toxicity. Eligible patients had squamous or adenocarcinoma of the cervix with measurable stage IVB or recurrent, aged 20–75 years, Eastern Cooperative Oncology Group performance status 0–2, prior platinum-containing regimen 0–1, and no prior taxane. The primary endpoint was overall response rate (ORR) by RECIST.

Results. 41 patients were enrolled, of which 39 were evaluable for analysis. 33 patients (84.6%) received prior radiotherapy. The confirmed ORR was 59% (95% CI, 43% to 75%); 5 patients (13%) achieved a complete response and median response duration was 5.2 months. The response rates for patients who had adenocarcinoma (n = 10) and prior platinum-based chemotherapy < 6 months (n = 7) were 40.0% and 0%, respectively. The median progression-free survival and overall survival times were 5.3 and 9.6 months, respectively. The most frequent grade 3 or 4 adverse events were neutropenia (79%), anemia (46%), thrombocytopenia (15%), and fatigue (8%). No treatment-related death was seen.

Conclusions. TC seemed to be feasible and effective similar to other cisplatin-based doublets for the treatment of metastatic or recurrent cervical cancer. Phase III trial is warranted to establish the clinical benefits of this combination.

Introduction

Cervical cancer remains the second most common cause of death due to cancer in women worldwide [1]. In the developed countries as well, a third of women with cervical cancer die of uncontrolled disease. Most women who have metastatic disease, including those with International Federation of Gynecology and Obstetrics (FIGO) stage IVB, or a local recurrence following radiotherapy are unfortunately no longer amenable to curable surgery or radiotherapy. In such circumstances, systemic chemotherapy is an important treatment option [2].

Historically, the most active single agent for advanced or recurrent cervical cancer has been cisplatin (P). However, its response rate (RR) has been generally low, varying from 17% to 38% with response duration of 3 to 6 months [3–6]. In order to improve prognosis, various studies have evaluated the survival benefit of adding other cytotoxic agents to P. Paclitaxel (T) as a single agent is moderately active against squamous cell carcinoma (SCC) (RR, 17%) [7]. One of the randomized controlled trials (RCT) performed by the Gynecologic Oncology Group (GOG) compared the combination of paclitaxel and cisplatin (TP) with single-agent cisplatin, and demonstrated a doubling of RR, median progression-free survival (PFS), and tolerable toxicity in patients with advanced or recurrent SCC [8]. Moreover, T is the most effective against non-SCC (adenocarcinoma and adenosquamous cell carcinoma) (RR, 31%) [9], of which the incidence now constitutes a larger percentage of the cervical cancer burden. TP is thus considered to be a superior chemotherapeutic regimen for both incurable SCC and non-SCC of the cervix. However, paclitaxel in this combination is given over 24-hour to reduce neurotoxicity and requires an inpatient hospital stay for each cycle. Therefore, equivalent or more effective and more feasible

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regimens are desperately needed to increase survival and improve quality of life (QoL).

Carboplatin (C) 340–400 mg/m^2 every 4 weeks, as a single agent, was evaluated to have less activity (RR, 15–28.2%) than cisplatin [10–12]. However, different from cisplatin, the dose of C should be calculated according to renal function [13]. In addition, the reduced nephrotoxicity and neurotoxicity of C enable a 3-hour administration of paclitaxel in the combination (TC) [14] without hydration in the outpatient setting. Moreover, paclitaxel has platelet-sparing activity in TC, which can mitigate the dose-limiting toxicity of C [15]. Although GOG 158 trial documented the therapeutic equivalency of TP and TC for treatment of ovarian cancer [16], experience with the use of TC in cervical cancer is limited. Therefore, we conducted the first prospective trial of TC for advanced or recurrent cervical cancer as a single-institutional phase II setting, and presented a promising feasibility and efficacy (RR: 61%) [17].

This multi-institutional phase II trial was designed to evaluate more precisely the efficacy and safety of the combination of paclitaxel and carboplatin for incurable SCC and non-SCC of the cervix, and to start a randomized phase III trial to evaluate the clinical benefits of TC compared with TP.

Materials and methods

Study design

This protocol was approved by the institutional review boards of each participating institution before patient enrollment. All patients provided written informed consent prior to enrollment.

Eligible patients had histologically proven stage IVB or recurrent carcinoma of the uterine cervix (squamous, adenocarcinoma, or adenosquamous) not amenable to curative treatment with surgery and/or radiotherapy, had at least one measurable disease by medical imaging according to Response Evaluation Criteria in Solid Tumors (RECIST; version 1.0) [18], were aged between 20 and 75 years, Eastern Cooperative Oncology Group performance status of 0, 1, or 2, had not received more than one prior platinum-based chemotherapy including concurrent chemoradiotherapy (CCRT), had no prior chemotherapy with taxanes, were at least from 4 weeks from any prior therapy, and had adequate hematologic (ANC$\geq$1500/μL, platelets$\geq$100,000/μL), renal (serum creatinine$\leq$2.0 mg/dL), hepatic (normal bilirubin, AST and alkaline phosphatase$\leq$2.5$\times$upper limit of normal (ULN)), and normal cardiac function.

Patients were excluded from study participation if they had bilateral hydronephrosis not amenable to decompression by either ureteral stents or percutaneous drainage, had neurological disturbance with functional disorder, had symptomatic central nervous system metastasis, had hypersensitivity to alcohol, or had active bacterial infection.

Assessments

Prior to each treatment cycle, patients were evaluated by history, physical examination including neurologic assessment, and blood test. Radiographic disease measurements were required after every two cycle to assess response according to RECIST version 1.0 criteria. Complete response (CR) and partial response (PR) not confirmed at $\geq$4 weeks were classified at stable disease (SD). Adverse events (AEs) were continuously evaluated according to the National Cancer Institute's common toxicity criteria [19]. The overall survival (OS) was defined as length of time from date of enrollment to death from any cause or the date of last contact. The progression-free survival (PFS) was defined as the period from study entry until disease progression, death, or the last date of contact. All patients who received at least one cycle of chemotherapy were assessed for efficacy and toxicity.

Treatment

Treatment consisted of paclitaxel 175 mg/m^2 for 3-hour given on day 1 followed by carboplatin at an area under the curve (AUC) of 5 mg/mL/min over given 1-hour on day 1, every 21 days. The carboplatin dose in milligrams was calculated using the Calvert formula [13], and glomerular filtration rate was estimated using the Cockcroft formula [20]. Patients received mandatory premedication with dexamethasone, diphenhydramine, and H_2 blocker before paclitaxel administration. Standard supportive care was used without prophylactic G-CSF.

Subsequent treatment cycles were not given unless ANC was $\geq$1500/μL, platelets were $\geq$75,000/μL, and all non-hematologic toxicities were resolved to grade 1 or lower (except for alopecia). The doses of both paclitaxel and carboplatin were reduced by 20% in case of platelet nadir<50,000/μL, any episode of febrile neutropenia, or grade 3 non-hematologic toxicity. In patients with grade 2 neuropathy, only the dose of paclitaxel was reduced by 20%. In patients with grade 3 neuropathy, treatment was delayed until resolution to grade 2 or less, and the paclitaxel dose was reduced by 40%. Once reduced, no dose escalation was allowed. Participants remained in the study for maximum of 6 cycles of chemotherapy. They were withdrawn from study if any of the following occurred: progressive disease (PD), grade 4 non-hematologic toxicity, or treatment delay of more than 3 weeks.

Statistical analysis

The primary endpoint of this trial was to assess the overall response rate (ORR) (CR + PR). The secondary endpoints were to assess AEs, PFS, and OS. This study was designed to evaluate ORR with a true probability of 50% considered clinically interesting against a null value of 30% deemed uninteresting. The desired probabilities of type I and II errors were 5% and 20%, respectively. The Simon's two-stage optimum design was applied [21]. This required approximately 28 evaluable patients to the first stage and a cumulative accrual of 39 to the second stage. The trial required more than 7/28 responses to proceed to the second stage and more than 15/39 to deem the regimen interesting. The Kaplan–Meier product-limit method was used to estimate PFS and OS.

Results

Patient characteristics

Forty-one eligible patients were enrolled at four participating clinical sites in Japan between January 2003 and December 2004. Two patients experienced grade 4 hypersensitivity reactions immediately after paclitaxel administration started at the first cycle, and their treatments were discontinued. The remaining thirty-nine patients received at least one cycle of protocol chemotherapy and were evaluable for treatment efficacy and toxicity. The patient characteristics are presented in Table 1. Median age was 47 years (range 29–68). The majority of patients had ECOG performance status of 1. Twenty-nine (74%) had squamous histology (SCC) and 10 (26%) had non-SCC (adenocarcinoma and adenosquamous). Before enrollment, 33 patients (85%) had received either whole pelvis irradiation alone or CCRT, and 31 (79%) had at least one target lesion outside of the previously irradiated field; lung, liver, lymph nodes, or bone metastases. Twenty-one patients (52%) had received either concurrent cisplatin plus radiotherapy as primary treatment for locally advanced disease or platinum-based chemotherapy for the first recurrence. Five patients (13%) had primary stage IVB disease and all of them received this protocol regimen as their primary treatment.

A total of 169 treatment cycles (median 4) were administered. Thirty patients (77%) received four or more cycles without disease progression and unacceptable toxicities, and 15 patients (38%) completed 6 cycles. Eleven patients (28%) required dose-delays mainly

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Table 1
Patient characteristics.

Characteristic	No. of patients	% of patients
Age, years		
Median	47	
Range	29–68	
ECOG performance status		
0	10	26
1	27	69
2	2	5
Histology		
Squamous cell carcinoma	29	74
Adenocarcinoma	8	21
Adenosquamous cell carcinoma	2	5
Prior radiation		
Yes	33	85
No	6	15
Target lesions outside of the prior irradiation field		
Yes	31	79
No	8	21
Platinum-free interval		
<6 months	7	18
≥6 months	14	36
No prior chemotherapy	18	46

Abbreviation: ECOG, Eastern Cooperative Oncology Group.

due to neutropenia or thrombocytopenia. Eight patients (20%) required a dose reduction for febrile neutropenia, thrombocytopenia, and/or neuropathy. There were no treatment-related deaths and no toxicity-related early treatment discontinuation.

Efficacy

The response assessment is presented in Table 2. ORR was 59% (95% CI 43%–75%). Five patients (13%) had CRs, 18 patients (46%) had PRs, and 9 patients (23%) had SDs. Therapeutic benefit (CR + PR + SD) was observed in 32 patients (82%, 95% CI 69%–95%). All patients who had non-SCC histology or all target lesions inside of the prior irradiation field did not experience CR. No response was observed in all patients who had received platinum-containing chemotherapy within the 6 months before enrollment.

Table 2
Response according to the patient characteristics.

Characteristics	No.					ORR	TBR
	Total	CR	PR	SD	PD	(%)	(%)
ECOG performance status							
0	10	3	5	1	1	80	90
1/2	29	2	13	8	6	52	79
Histology							
SCC	29	5	14	5	5	66	83
Non-SCC	10	0	4	4	2	40	80
Target lesions outside of the prior irradiation field							
Yes	31	5	14	7	5	61	84
No	8	0	4	2	2	50	75
Drug (platinum)-free interval							
<6 months	7	0	0	4	3	0	57
≥6 months	14	2	7	3	2	64	86
No prior chemotherapy	18	3	11	2	2	78	89
Total	39	5	18	9	7	59	82

Abbreviations: CR, complete response;
PR, partial response;
SD, stable disease;
PD, progressive disease;
ORR, overall response rate (CR + PR);
TBR, therapeutic benefit rate (CR + PR + SD);
ECOG, Eastern Cooperative Oncology Group;
SCC, squamous cell carcinoma.

The median duration of response was 5.2 months (range 1.6–72.7 months). The median PFS was 5.3 months (95% CI, 4.1–14.0 months; Fig. 1). The median OS was 9.6 months (95% CI, 6.8–20.0 months; Fig. 2).

Safety

AEs are presented in Table 3. Thirty-one patients (79%), all of whom had received prior pelvic irradiation, developed grade 3 or 4 neutropenia. Although this led to fever in 4 patients (10%), they recovered after treatment with oral antibiotics (ciprofloxacin) and developed no sepsis. Eighteen patients (46%) experienced grade 3 or 4 anemia, and a total of 8 patients received blood transfusions. No patient needed platelet transfusions. Any grade non-hematologic toxicities consisted primarily of neuropathy (74%), excluding alopecia (82%). Neuropathy was cumulative, but mild in almost patients (grade 1; 64%). Only one patient experienced grade 3 neuropathy and removed from the study. Two patients (5%) had fever without neutropenia (secondary to herpes). As a result, no evaluable patients required hospitalization during the protocol treatment.

Discussion

In this multi-institutional trial similar to a prior single-institutional trial [17], the combination of paclitaxel and carboplatin (TC) provided a promising response rate (RR) of 59%, which is better than those recorded in a recent pivotal phase III trial comparing four cisplatin-containing doublet combinations. In the GOG204 trial, paclitaxel plus cisplatin (TP) had a RR of 29%, while topotecan plus cisplatin had a 23% RR [22]. In a prior study (GOG179), topotecan plus cisplatin had a 27% RR and became the first combination regimen to show significantly longer overall survival (OS) than single-agent cisplatin [23]. Progression-free survival (PFS) was similar to that seen with TP, 5.3 months versus 5.8 months, respectively. OS of 9.6 months was not also remarkably inferior to that reported for TP (12.9 months).

Toxicities were predictable based upon the regimen, with neutropenia being the most common adverse event (AE) due to the limited reservation of bone marrow post pelvic irradiation. However, no patient developed sepsis and needed platelet transfusions. Neuropathy due to paclitaxel was common and usually mild, as only one patient was removed from the treatment protocol because of this. The

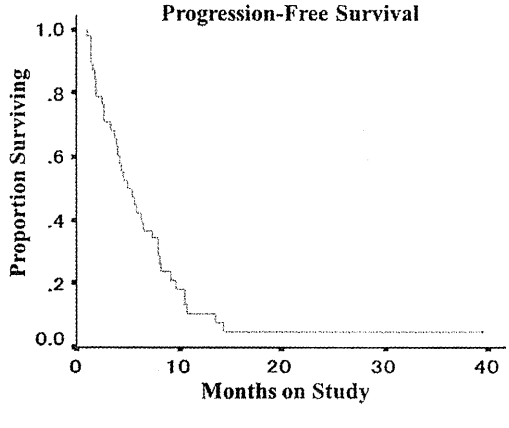

Fig. 1. Kaplan–Meyer survival curve of progression-free survival (PFS).

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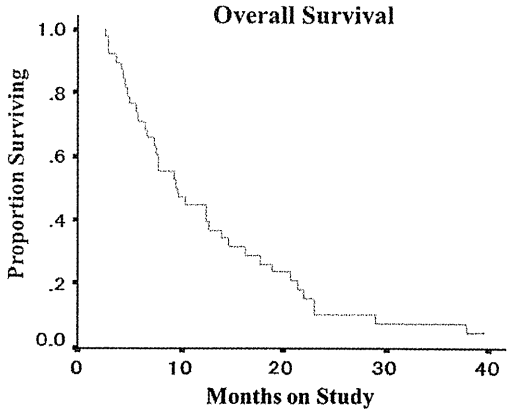

Fig. 2. Kaplan–Meyer survival curve of overall survival (OS).

enrolled patients experienced no grade 4 non-hematologic toxicity and no treatment-related death, and required no hospitalization during the protocol treatment. We thus showed that TC was well-tolerated and feasible regimen in outpatient setting, and that hospitalization periods as a surrogate for QoL were remarkably improved for patients throughout the treatment. These findings are especially important when treating patients in a palliative setting.

In the current trial, histology or having target lesions inside of the prior irradiated field appeared to be negatively prognostic for ORR. On the other hand, we also showed that no response was significantly observed in patients with prior platinum administration within the past 6 months. In the GOG179 and GOG204, as well as, it is possible that prior CCRT is associated with an increased risk of death, and Tanioka et al. reported that platinum-free interval (PFI) had prognostic value for second platinum therapy [24]. One hypothesis that accounts for these decreased efficacies is drug resistance to platinum in such patients in the same way of ovarian cancer [25]. Therefore, non-cross resistant drugs for platinum will be also required.

This trial demonstrates that combination therapy with paclitaxel and carboplatin is promisingly effective and reasonably safety in treating patients with advanced and recurrent cervical cancer. Furthermore, this regimen appears to be remarkably convenient (day 1 of a 21-day cycle in the outpatient clinic) for treating patients in a palliative setting. Based upon the overall response rate and survival data, we have already conducted a phase III trial of paclitaxel plus carboplatin compared with paclitaxel plus cisplatin in the efficacy and improved QOL for incurable SCC or non-SCC cervical cancer. The study protocol was designed by the Gynecologic Cancer Study Group (GCSG) of the Japan Clinical Oncology Group (JCOG), approved by the Protocol Review Committee of the JCOG on January 12, 2006, and activated on February 21, 2006. This multi-institutional (30 specialized institutions), randomized controlled trial (JCOG0505) was registered at the UMIN Clinical Trials Registry as C000000335 [26].

Table 3
Adverse events.

Toxicity	Maximum grade (no. of patients)				Grades 3–4	
	1	2	3	4	No.	%
Hematologic						
Neutropenia	4	4	11	20	31	79
Febrile neutropenia	–	–	4	0	4	10
Anemia	8	10	14	4	18	46
Thrombocytopenia	6	3	6	0	6	15
Non-hematologic						
Allergy	3	3	0	0	0	0
Fatigue	13	4	3	0	3	8
Alopecia	3	29	–	–	–	–
Nausea/vomiting	13	7	1	0	1	3
Diarrhea	4	2	2	0	2	5
Arthritis/myalgia	8	8	1	0	1	3
Neuropathy	25	3	1	0	1	3
Infection	0	2	0	0	0	0

Conflict of interest statement

Dr. Yasuhiro Fujiwara declares research grants from Pfizer, GSK, Chugai, Eisai, Daiichi Sankyo, Taiho Pharmaceutical, and Nihon Kayaku.

All other authors have no conflict of interest to declare.

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Use of squamous cell carcinoma antigen as a biomarker of chemotherapy response in patients with metastatic cervical carcinoma

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ABSTRACT

Objective: To examine the use of squamous cell carcinoma antigen (SCCA) as a biomarker of chemotherapy response in patients who underwent chemotherapy for metastatic cervical carcinoma.
Study design: The study population consisted of patients who underwent first-line chemotherapy for metastatic cervical carcinoma between 1999 and 2009. SCCA levels were serially measured before, during and after chemotherapy. Radiographic responses were evaluated according to the criteria of the World Health Organization. A logistic model was used to determine the best prediction model, and internal and external validation of the prediction model were performed to compare the areas under the receiver operating characteristic curves (AUCs).
Results: In total, 55 patients were included in the analysis. Data for 32 patients enrolled in various clinical trials were used to develop the prediction model. Patients who achieved a radiographic response showed a significant decline in SCCA levels between the second and third cycles of chemotherapy, whereas patients who did not achieve a radiographic response showed constant SCCA levels over the same period. The prediction model was developed on the basis of changes in the SCCA level between the second and third cycles of chemotherapy (AUC = 0.832) and the baseline SCCA level. The AUC after external validation, calculated using the data of the clinical practice population ($n = 22$), was 0.871.
Conclusions: A response to chemotherapy was possible for patients in whom SCCA levels declined between the second and third cycles of chemotherapy.

1. Introduction

Squamous cell carcinoma antigen (SCCA) is often used as a tumour marker in patients with cervical carcinoma. It is a 45–55-kDa protein isolated from cervical carcinoma or normal squamous epithelium, and its sensitivity in patients with stage IV cervical squamous cell carcinoma has been reported to be 89% [1]. An elevated pretreatment level of SCCA has been identified as a prognostic factor in early-stage cervical carcinoma, and monitoring of SCCA levels after neoadjuvant chemotherapy provides information regarding the response to chemotherapy [2,3]. In the case of patients with recurrent or metastatic cervical carcinoma, an association has been reported between the response to chemotherapy and declining SCCA levels [4].

SCCA levels have been studied in depth and have been reported to predict the recurrence of cervical cancer after surgery and disease prognosis. SCCA has higher sensitivity than cytokeratins, a multigene family of 20 related polypeptides, and SCCA is therefore a better candidate for monitoring purposes [4]. Monitoring the levels of SCCA in metastatic cervical carcinoma patients treated with chemotherapy is a non-invasive technique, and may benefit such patients in the clinical setting. However, no validated data are currently available for SCCA in this context. Moreover, no other established tumour markers are available to monitor the response to chemotherapy in patients with metastatic cervical carcinoma. Further, radiographic determination of the response to chemotherapy in conjunction with evaluation of tumour marker levels is essential for accurate prognosis. Therefore, the routine measurement of SCCA levels without radiography is not currently recommended. This paper reports the use of SCCA as a biomarker of chemotherapy response in patients with metastatic cervical carcinoma.

2. Patients and methods

2.1. Patient population

In this study, two patient populations were used to evaluate the correlation between SCCA levels and the documented responses.

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One population was from a clinical practice and comprised patients: (a) who underwent first-line chemotherapy for metastatic cervical carcinoma between October 1999 and April 2009 at the National Cancer Centre Hospital; (b) whose histological examination showed adenosquamous cell carcinoma or squamous cell carcinoma; (c) who had measurable lesion(s) according to the criteria of the World Health Organization (WHO) [5]; and (d) whose SCCA levels were measured before and during chemotherapy. Metastatic cervical carcinoma was defined as a primary lesion with distant metastasis or recurrence after local therapy, including radiotherapy or surgery.

The other population comprised patients who were enrolled in phases II and III clinical trials for metastatic cervical carcinoma, and who fulfilled the following criteria: age $\geq$18 years; Eastern Cooperative Oncology Group performance status $\leq$2; no active infections or interstitial pneumonitis; measurable disease according to the WHO criteria [6]; and adequate organ function. In addition, all the patients in this population agreed to provide their informed consent for the trials. In the study period, all the patients who met the eligibility criteria for each trial were given basic information about the trials. Patients who did not meet the criteria or who refused to participate in the trials received chemotherapy regimens that were considered standard at the time. Results of the clinical trials performed to confirm the survival benefit of chemotherapy or to examine the response rate have not been published to date.

2.2. Chemotherapy regimens

The major chemotherapy regimens used for this study were as follows: (a) paclitaxel 135 mg/m^2 on Day 1 and cisplatin 50 mg/m^2 on Day 2 every 3 weeks (q3w); (b) paclitaxel 175 mg/m^2 on Day 1 and carboplatin [area under the receiver operating characteristic (ROC) curve (AUC) 5 or 6] on Day 1 q3w; (c) cisplatin 14 mg/m^2 on Days 1–5, bleomycin 7 mg/m^2 on Days 1–5, mitomycin-C 7 mg/m^2 on Day 5, and vincristine 0.7 mg/m^2 on Day 5 q3w; and (d) irinotecan 60 mg/m^2 on Days 1, 8 and 15, and carboplatin (AUC 5) on Day 1 q3w.

2.3. SCCA assay

For all the patients, complete blood cell counts, blood chemistry parameters and serum tumour marker levels were measured using blood samples obtained within 1 week of initiating chemotherapy. Samples were obtained on Day 1 of each cycle of chemotherapy to avoid cytolytic effects, and were assayed immediately in the Clinical Laboratory Division of the National Cancer Centre Hospital. Serum SCCA levels were measured using an enzyme immunoassay (based on the sandwich principle; Dynapack, Abbott Japan Co. Ltd., Tokyo, Japan). Quality control and calibration for tumour marker measurement were performed using the commercial reference control sera. The upper normal limit for SCCA was 1.5 ng/ml.

2.4. Statistical analyses

The response to treatment [i.e. complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD)] was determined for all patients based on the WHO criteria. Baseline SCCA levels, SCCA levels on Day 1 of the second and third cycles of chemotherapy in the combined populations, and percentage changes in the SCCA levels between CR/PR and SD/PD groups in the combined populations were compared using the Wilcoxon test. The frequency distribution of elevation of baseline SCCA levels between the two groups was compared using Fisher's exact test. Furthermore, correlation between changes in the SCCA levels and the documented response (CR/PR or SD/PD) was evaluated through the development and validation of a model predicting the documented responses on the basis of SCCA levels.

To develop and validate the prediction model, information regarding the documented response (CR/PR or SD/PD) and SCCA levels (baseline levels and levels on Day 1 of the second and third cycles of first-line chemotherapy) was obtained from the medical charts of the 55 patients. Percentage changes in the SCCA levels were calculated using the following formulae:

$$\text{SCCA level I} = \frac{\text{level on Day 1 of the second cycle of chemotherapy} - \text{initial level}}{\text{initial level}}$$

$$\text{SCCA level II} = \frac{\text{level on Day 1 of the third cycle of chemotherapy} - \text{level on Day 1 of the second cycle of chemotherapy}}{\text{level on Day 1 of the second cycle of chemotherapy}}$$

The prediction model for the documented responses was developed on the basis of a multivariate logistic regression model using the data of SCCA levels of the 32 patients enrolled in clinical trials. Several linear combinations of marker levels included in the logistic model were selected from SCCA I, SCCA II and baseline SCCA levels. To measure the accuracy of the prediction model, the AUC for each candidate prediction model was calculated by the bootstrap method (300 bootstrap replicates) as internal validation in order to reduce bias [7]. The overall misclassification rate (OMR) of the model was also calculated. The model with the maximum AUC was determined to be the best prediction model. If several models with the same AUC were obtained, the more parsimonious model with lower OMR values was regarded as the better model.

After development of the prediction model, it was verified using an external validation dataset, which comprised an independent consecutive series of 23 patients who had received first-line chemotherapy in clinical practice at the National Cancer Centre Hospital between January 1999 and March 2009. The AUC and OMR values for the external validation dataset were calculated to evaluate the performance of the prediction model. Kaplan–Meier survival curves were drawn, and the log-rank test was used to detect the differences. $p < 0.05$ was taken to indicate statistical significance. All statistical analyses were performed using SAS Version 9.1 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Patient characteristics

Patient characteristics are summarized in Table 1, and were similar in each cohort.

3.2. Difference in SCCA levels between CR/PR and SD/PD groups

Table 2 shows the SCCA levels in the CR/PR and SD/PD groups. Differences in the SCCA levels on Day 1 of the third cycle between the two groups were significant ($p = 0.002$), although the differences in the baseline SCCA levels and those on Day 1 of the second cycle between the two groups were not significant. The results shown in Table 2 indicate that the SCCA levels in the CR/PR

Table 1
Patient characteristics.

Characteristics	Population in clinical trials	Population in clinical practice	*p*-Value[a]
Number of patients	32	23	
Age in years, mean (SD)	54.8 (11.9)	53.4 (13.5)	0.675
Performance status, *n* (%)			0.223
0–1	30 (93.8)	19 (82.6)	
>2	2 (6.3)	4 (17.4)	
Disease status, *n* (%)			0.116
Recurrence	30 (93.8)	18 (78.3)	
Primary metastases	2 (6.3)	5 (21.7)	
Metastatic sites, *n* (%)			
Subclavian lymph nodes			0.383
Yes	5 (15.6)	1 (4.3)	
No	27 (84.4)	22 (95.7)	
Mediastinal lymph nodes			0.686
Yes	5 (15.6)	2 (8.7)	
No	27 (84.4)	21 (91.3)	
Cervical lymph nodes			1.000
Yes	1 (3.1)	1 (4.3)	
No	31 (96.9)	22 (95.7)	
Pelvic lymph nodes/local recurrence			1.000
Yes	19 (59.4)	14 (60.9)	
No	13 (40.6)	9 (39.1)	
Lung			0.064
Yes	5 (15.6)	9 (39.1)	
No	27 (84.4)	14 (60.9)	
Liver			1.000
Yes	4 (12.5)	2 (8.7)	
No	28 (87.5)	21 (91.3)	
Bone			0.068
Yes	0 (0.0)	3 (13.0)	
No	32 (100.0)	20 (87.0)	
Chemotherapy regimen, *n* (%)			0.026
Cisplatin based	8 (25.0)	4 (17.4)	
Carboplatin based	17 (53.1)	19 (82.6)	
Other	7 (21.9)	0 (0.0)	
Response, *n* (%)			0.331
Complete response	5 (15.6)	2 (8.7)	
Partial response	12 (37.5)	9 (39.1)	
Stable disease	14 (43.8)	8 (34.8)	
Progressive disease	1 (3.1)	4 (17.4)	

SD, standard deviation.
[a] Univariate analysis: *t*-test and Fisher's exact test (two-sided) were performed to calculate the mean differences or proportional differences between the two groups.

group decreased from the second cycle through the third cycle, while those in the SD/PD group did not.

3.3. Correlation between SCCA levels and documented responses

Several prediction models were evaluated as candidates on the basis of the AUCs calculated by the bootstrap method. Seven combinations of markers and AUCs are summarized in Table 3. As shown in Table 3, the combination of SCCA level II and elevation of baseline SCCA level contributed more to the prediction of CR/PR than the other combinations. The best prediction model, with an AUC of 0.832, defined the probability of PR/CR as follows: probability of PD = $e^x/(1+e^x)$, where e is the base of the natural logarithm and $x = (0.91 - 3.74 \times$ SCCA level II $- 1.98 \times$ elevated baseline SCCA level). Furthermore, the *p*-values of the Wald Chi-squared tests for coefficients of SCCA level II and elevated baseline SCCA level were 0.008 and 0.097, respectively. The prediction rule was as follows: a calculated response probability of $\geq$0.40 corresponded to CR/PR, while a calculated response probability of <0.40 corresponded to SD/PD. The OMR of the model, calculated using the development data, was 0.188. The model was verified using the external validation dataset of an independent consecutive series of 23 patients who were being treated in a clinical practice. The ROC curve is shown in Fig. 1, wherein the AUC was 0.871. Additionally, the 23 patients

Table 2
Squamous cell carcinoma (SCC) antigen levels in complete response/partial response (CR/PR) and stable disease/progressive disease (SD/PD) groups.

	CR/PR group	SD/PD group	*p*-Value[a]
Number of patients	28	27	
Baseline SCC level, median (range)	8.5 (0.5–181.0)	9.0 (0.2–196.0)	0.674
Patients with elevated baseline SCC level, *n* (%)	20 (71.4)	24 (88.9)	0.177
SCC level on Day 1 of the second cycle of chemotherapy, median (range)	3.3 (0.5–100.6)	3.5 (0.3–308.0)	0.096
SCC level on Day 1 of the third cycle of chemotherapy, median (range)	1.3 (0.1–23.0)	3.3 (0.6–355.0)	0.002
SCC level I, median (range)	−0.26 (−0.96 to 1.60)	−0.26 (−0.85 to 1.33)	0.033
SCC level II, median (range)	−0.41 (−0.92 to 0.33)	0.03 (−0.76 to 3.27)	0.001

SCCA level I = (level on Day 1 of the second cycle of chemotherapy − initial level)/initial level. SCCA level II = (level on Day 1 of the third cycle of chemotherapy − level on Day 1 of the second cycle of chemotherapy)/level on Day 1 of the second cycle of chemotherapy.
[a] Univariate analysis: Wilcoxon test and Fisher's exact test (two-sided) were performed to calculate the rank differences or proportional differences between the two groups.

Table 3
Seven combinations of markers and their areas under the receiver operating characteristic curve (AUCs).

SCCA level I	SCCA level II	Elevation of baseline SCCA level	AUC
×			0.557
	×		0.811
		×	0.532
×	×		0.777
×		×	0.601
	×	×	0.832
×	×	×	0.794

SCCA level I = (level on Day 1 of the second cycle of chemotherapy – initial level)/initial level. SCCA level II = (level on Day 1 of the third cycle of chemotherapy – level on Day 1 of the second cycle of chemotherapy)/level on Day 1 of the second cycle of chemotherapy.

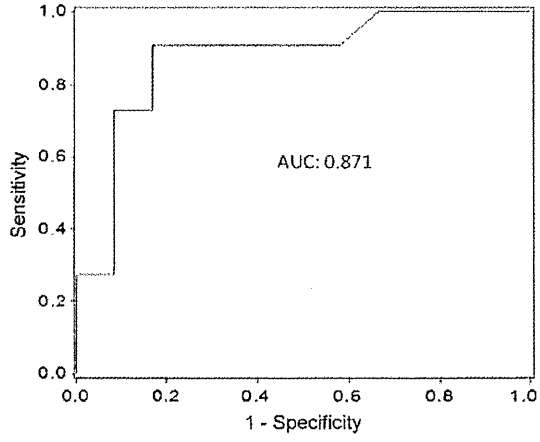

Fig. 1. Receiver operating characteristic curve for the external validation dataset. AUC, area under the curve.

being treated in a clinical practice were categorized into two groups (predicted CR/PR and SD/PD groups) based on the predictive model. Fig. 2 shows the mean SCCA level in each group (mean value for each day is connected with a line), and the individual SCCA levels at baseline and Day 1 of the second and third cycles of chemotherapy. The mean SCCA level in the predicted SD/PD group decreased on Day 1 of the second cycle but increased on Day 1 of the third cycle, although it decreased monotonically in the predicted CR/PR group.

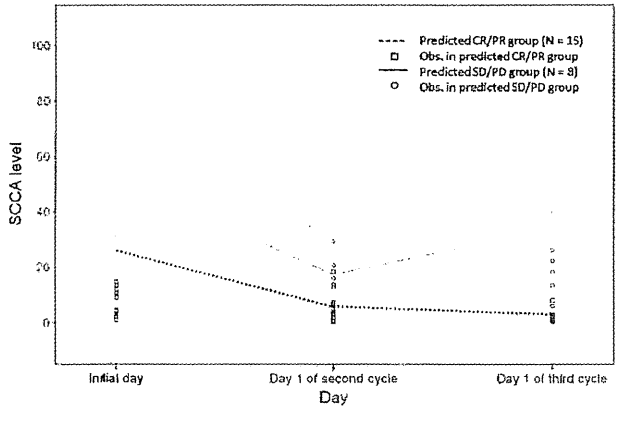

Fig. 2. Squamous cell carcinoma antigen (SCCA) levels in the predicted complete response/partial response (CR/PR) and stable disease/progressive disease (SD/PD) groups. Obs. observed.

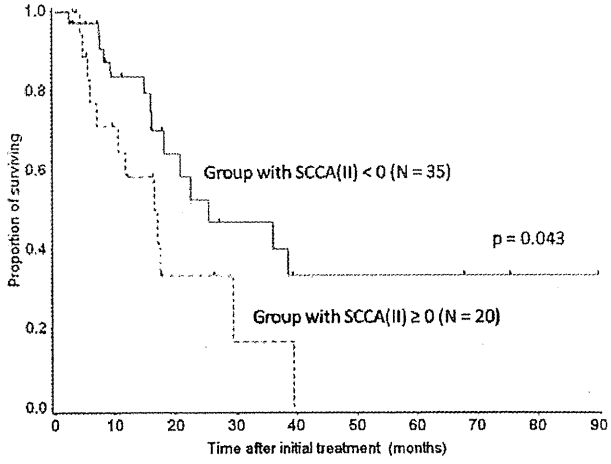

Fig. 3. Overall survival in patients classified by category. Squamous cell carcinoma antigen (SCCA) level II = (level on Day 1 of the third cycle of chemotherapy – level on Day 1 of the second cycle of chemotherapy)/level on Day 1 of the second cycle of chemotherapy.

Based on the value of SCCA level II, 55 patients were categorized into two groups (i.e. SCCA level II <0 and SCCA level II ≥0). Thirty-five patients had SCCA level II <0, and the remaining 20 patients had SCCA level II ≥0. Overall survival, defined as the time from initial treatment to death due to any cause, was compared between the two groups by the log-rank test. The Kaplan–Meier curve for overall survival of the two groups is shown in Fig. 3. The difference in the survival curves between the two groups was significant ($p = 0.043$).

4. Comments

A model was developed to predict the response to chemotherapy based on serum SCCA levels in patients with metastatic cervical carcinoma. First, a model using data of SCCA levels in patients who participated in clinical trials was developed. This model was validated using data from patients with metastatic cervical carcinoma who received chemotherapy in clinical practice. In the SCCA model, longer survival was noted for patients who achieved PR or CR compared with patients with SD or PD. The results suggest that the patients who had baseline SCCA levels above the normal range and in whom the SCCA level declined between the second and third cycles of first-line chemotherapy could achieve a partial or complete radiographic response.

Previously, SCCA levels have been evaluated to determine the response to chemotherapy. These studies have suggested a correlation between the response to radiotherapy or chemotherapy and changes in SCCA levels [8–11]. Interestingly, Hong et al. reported that persistent elevation of SCCA levels after 2–3 months of radiotherapy suggested a higher chance of treatment failure, which agrees with the present results [9]. Other reports have attempted to determine the relationship between pretreatment SCCA levels and the prognosis or treatment response [9,11–14]. However, although certain studies have analysed the serial changes in SCCA levels in relation to prognosis [9,11,13,14], no study has developed and validated a model to predict the response to chemotherapy in patients with advanced cervical carcinoma.

Gadducci et al. reviewed several reports targeting SCCA levels for monitoring cervical carcinoma, and reported that monitoring SCCA in the follow-up of early-stage cervical cancer does not appear to improve the clinical outcome. Furthermore, they indicated that the specificity of this marker is not optimal [4]. However, monitoring the levels of SCCA is a non-invasive and non-radioactive technique, which is a major advantage. If data for SCCA

levels in relation to response to chemotherapy are developed and validated, similar to those of CA125 for ovarian cancer [15], it can be a useful tool for predicting progression. The SCCA model encourages clinicians to perform radiography. In ovarian cancer, early treatment of relapse on the basis of a raised CA125 concentration or delayed treatment by radiography has no impact on survival [16]. SCCA levels in cervical cancer should be examined prospectively based on similar logic.

Due to the small study sample, the model used in this study to predict either SD/PD or PR/CR had a prediction rate of 40%, although the OMR and AUC were somewhat high. Validated data for SCCA and its prognostic value are limited. Further prospective studies are needed to establish the utility of SCCA.

In conclusion, a correlation was found between the decline in SCCA levels between the second and third cycles of chemotherapy and the response to chemotherapy in patients with metastatic cervical carcinoma.

Conflict of interest

None declared.

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Development of a mouse model for lymph node metastasis with endometrial cancer

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Controlling lymph node metastasis is currently a key issue in cancer therapy. Lymph node metastasis is one of the most important prognostic factors in various types of cancers, including endometrial cancer. Vascular endothelial growth factor-C (VEGF-C) plays a crucial role in lymphangiogenesis, and is implicated to play an important role in lymph node metastasis. To evaluate the role of VEGF-C in lymph node metastasis, we developed an animal model by using an endometrial cancer cell line, HEC1A. This cell line is not invasive by nature and secretes moderate amounts of VEGF-C; intrauterine injection of HEC1A cells into Balb/c nude mice resulted in uterine cancer with lymph node metastasis after 8 weeks. To analyze the contribution of VEGF-C to lymph node metastasis, its corresponding gene was stably introduced into HEC1A cells (HEC1A/VEGF-C), which then produced more than 10 times the amount of VEGF-C. The number of lymph node metastases was significantly higher in HEC1A/VEGF-C cells than in HEC1A cells (3.2 *vs* 1.1 nodes/animal, respectively). Augmented lymphangiogenesis was observed within tumors when HEC1A/VEGF-C cells were inoculated. These results indicate that VEGF-C plays a critical role in lymph node metastasis, in addition to serving as a platform to test the efficacy of various therapeutic modalities against lymph node metastasis. (*Cancer Sci* 2011; 102: 2272–2277)

Endometrial cancer is one of the most common gynecological malignancies, and the fourth most common malignancy.[1] The overall prognosis of endometrial cancer is considered to be better than that for other types of gynecological malignancies, because the disease can be detected in its early stages. However, the prognosis of patients with advanced stages of endometrial cancer is still poor, owing to the lack of effective treatment modalities. Furthermore, the overall survival rate for such patients has not improved over the past 30 years.[1] One of the most important prognostic factors in endometrial cancer is lymph node metastasis.[2,3] Therefore, it is vital to develop new treatment modalities that focus on lymph node metastasis.

The factors involved in lymphangiogenesis and lymph node metastasis were recently elucidated; it has become clear that vascular endothelial growth factor (VEGF)-C is a significant contributor.[4–6] Vascular endothelial growth factor-C is a 38-kDa glycoprotein that acts through a tyrosine kinase-type receptor, VEGF receptor 3 (VEGFR3). It is suggested that during malignancy, VEGF-C produced by tumor and/or interstitial cells promotes lymph node metastasis.[7] Vascular endothelial growth factor-C expression in uterine endometrial carcinoma was found to be related to both lymphatic vessel invasion and lymph node metastasis in a study with 228 surgical cases of endometrial cancer.[4]

The development of an adequate animal model is critical for facilitating research on lymph node metastasis. Therefore, we aim to develop a suitable animal model by using endometrial cancer cells.

Materials and Methods

Cells and plasmids. Human endometrial cancer cell lines, HEC1A and HEC50B, were obtained from the Japanese Collection of Research Bioresources; the cell lines were authenticated through the multiplex PCR method, using short tandem repeats,[8] and were maintained as described previously.[9,10] The Ishikawa cell line (clone 3H12) was a gift from Dr. M. Nishida (Department of Obstetrics and Gynecology, National Hospital Organization, Kasumigaura Medical Center, Ibaraki, Japan), and was maintained as described previously.[11] The VEGF-C sequence was obtained by PCR, using the following primer set against human placental cDNA: forward, 5′-ATGC-ACTTTGCTGGGCTTCTT-3′; reverse, 5′-CAATCTTAGC-TCATTTGTGGTCT-3′. The VEGF-C expression plasmid, pCMV–VEGF-C–internal ribosome entry site (IRES)–blasticidin S-resistance (*bsr*) gene, was constructed by inserting the VEGF-C sequence into the *Eco*RI and *Xba*I sites of pCMV–IRES–*bsr*.[12]

Development of stably-transduced cells. The VEGF-C expression plasmid, pCMV–VEGF-C–IRES–*bsr*, and the control, pCMV–luciferase (LUC)–IRES–*bsr*,[12] were introduced into HEC1A cells by using the standard calcium phosphate method. The structures of these plasmids are shown in Figure 1. According to our previous experiments, introducing pCMV–LUC–IRES–*bsr* does not alter the growth, migration, invasive capacity, anticancer drug sensitivity, or the radiosensitivity of cells.[13]

Cells were selected in the presence of 10 μg/mL blasticidin S hydrochloride (Funakoshi, Tokyo, Japan) for 2 weeks, and the resistant cells were collected as HEC1A/VEGF-C and HEC1A/LUC.

Vascular endothelial growth factor-C quantification in culture supernatant. The culture medium was replaced by fresh medium without serum. After 48 h of culturing, the supernatants of each cell line (HEC1A, HEC1A/LUC, HEC1A/VEGF-C, HEC50B, and Ishikawa 3H12) were collected and subjected to VEGF-C analysis, using a Quantikine human ELISA kit (R&D Systems, Minneapolis, MN, USA).

***In vitro* cell growth kinetics.** The HEC1A, HEC1A/VEGF-C, and HEC1A/LUC cells were dispersed so that 1×10^5 cells were present in each well of 3.5-cm plastic dishes. After culturing, the cells were dislodged using 0.05% trypsin–EDTA every 24 h to determine the number of cells by using a hemocytometer.

***In vivo* tumor growth by subcutaneous inoculation.** Five- to 6-week-old female Balb/c nude mice (CLEA Japan, Tokyo, Japan) were used for the tumor growth experiments. All animal experiments were conducted according to the institutional and national guidelines for animal experiments. The HEC1A,

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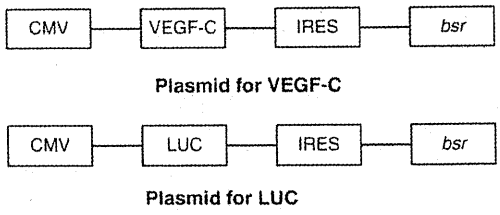

Fig. 1. Structure of the plasmids used in this study. Plasmids encoding vascular endothelial growth factor-C (VEGF-C) driven by the CMV promoter were used for cellular transduction. Plasmids encoding luciferase (LUC) were used as controls. *bsr*, blasticidin S-resistance gene; IRES, internal ribosome entry site.

HEC1A/VEGF-C, and HEC1A/LUC cells were implanted dorsally under the skins of the mice at 5×10^6 cells/site. Tumor volume was estimated using the formula: $0.5 \times L \times W^2$, where L and W indicate length and width in millimeters, respectively ($n = 5$).[14]

***In utero* transplantation of tumor cells.** Five- to 6- week-old female BALB/c nude mice (CLEA Japan) were used for the *in utero* experiments. A diagram of the injection procedure is shown in Figure 2. A laparotomy with a transverse incision was performed under general anesthesia, followed by ligation of the openings of the uterus at three locations, using 4-0 Vicryl (Ethicon, New Brunswick, NJ, USA). Tumor cells (5×10^6 cells) suspended in 50 μL PBS were injected into the uterine cavities by using a syringe with a 29-gauge needle. The left and right uterine cavities received similar volumes of cell suspension. The incisions were closed after the uterine tubes were inspected for proper enlargement and the absence of leakage. Uterine involvement and tumor development, especially lymph node metastasis, were evaluated periodically by laparotomy after the mice were killed.

Histological analysis of the animals. The animals were killed as scheduled, and all of the abdominal, thoracic, and retroperitoneal organs were inspected macroscopically. The metastatic lesions, uteri, and other organs showing possible signs of metastasis were microscopically evaluated for tumor progression. To estimate lymph node metastasis by size, recognizable lymph nodes were excised, and the presence of tumor metastasis was evaluated by histology in the first and second animal series. In the third and fourth animal series, the number of lymph node metastases was counted by enumerating lymph nodes larger than 3 mm in longitudinal diameter and evaluating them histologically.

Lymphangiogenesis in the subcutaneous tumor. At 2 weeks after the subcutaneous transplantation of corresponding cells (5×10^6 cells per animal) into the back, the mice ($n = 4$) were killed, and the subcutaneous tumors were excised. After fixation

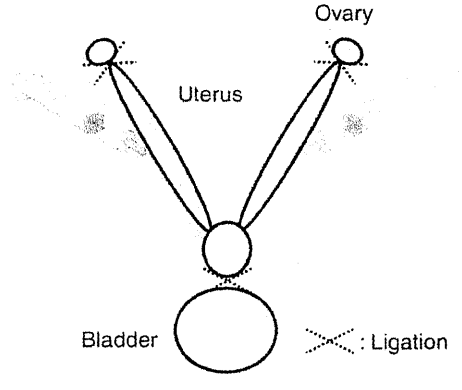

Fig. 2. Tumor cell injection procedure. After laparotomy under general anesthesia, all ends of the uterus were closed by ligation, ensuring the settlement of tumor cells within the uterine cavity. Following ligation, the cells were injected by puncturing the uterine wall. Abdominal incision was sutured after confirming that there was no leakage of cell suspension from the uterus.

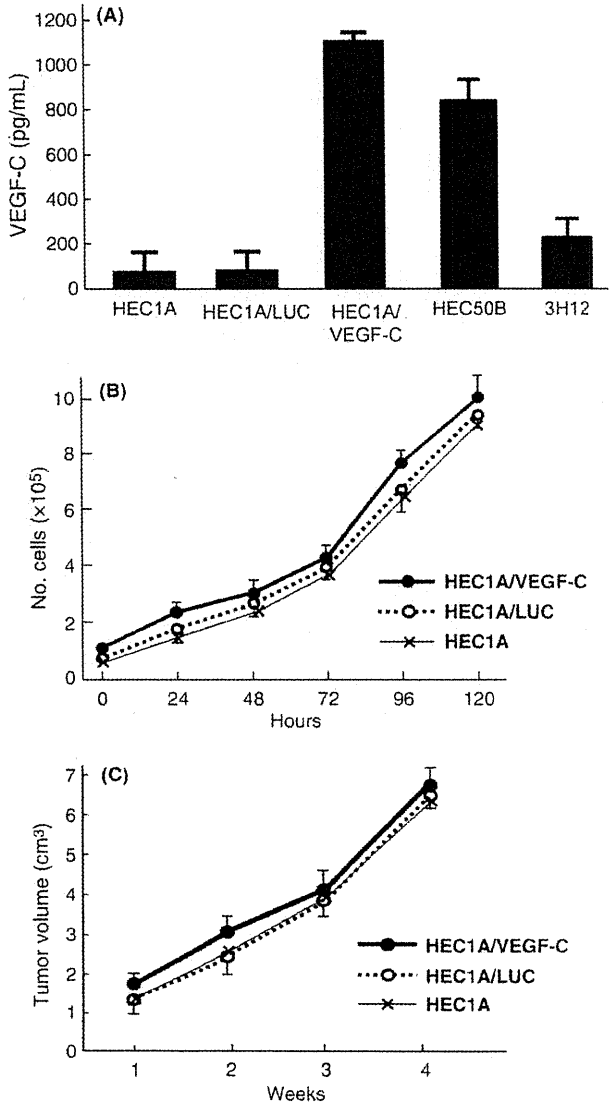

Fig. 3. (A) Vascular endothelial growth factor-C (VEGF-C)- or luciferase (LUC)-encoding plasmids were introduced into HEC1A cells by calcium phosphate transfection. Resultant cells were maintained, and the culture media were replaced with serum-free media upon confluence. After 48 h, the VEGF-C concentration of culture supernatants was determined by ELISA. Supernatant from the HEC1A/VEGF-C cells exhibited 10 times the concentration of VEGF-C, compared to the HEC1A or HEC1A/LUC cells. (B) Cells were dispersed at a concentration of 1×10^5/well in six well plates and were subsequently cultured. Cells were dislodged and counted after 24 h. There were no differences in the growth among these cell lines. (C) Growth of subcutaneous tumors after injection. Cells were subcutaneously injected into Balb/c nude mice (5×10^6 cells/site), and the tumor volumes were determined during follow up. No differences were found between these cells *in vivo*.

of the tumors in 4% paraformaldehyde, frozen sections were sliced, and antigen enhancement was done by heating the sections at 121°C in sodium citrate buffer (0.01 mol/L, pH 6.0) for 10 min, and endogenous peroxidase was blocked with 3% H_2O_2. The sections were incubated overnight at 4°C with a 1:500 dilution of anti-VEGFR3 antibody (Abcam, Cambridge, UK) as the primary antibody recognizing lymphatic endothelial cells, and then reacted with the secondary antibody, that is, the peroxidase-conjugated antirat antibody (Simple Stain Mouse MAX-PO, Rat; Nichirei, Tokyo, Japan) at room temperature for 30 min, followed by color development with diaminobenzidine. The number of newly-formed lymph vessels was counted under a light microscope at ×20 magnification. A single section was prepared per mouse in four animals per group, and new lymph vessels were counted in the four sections and averaged.

Verification. All *in vitro* experiments were performed at least three times.

Results

***In vitro* production of VEGF-C and cell growth kinetics.** Culture supernatants of the HEC1A and HEC1A/LUC cells showed similar VEGF-C concentrations, whereas the HEC1A/VEGF-C cells produced much greater concentrations of VEGF-C (Fig. 3A). There were no differences in the growth properties of the HEC1A/LUC and HEC1A/VEGF-C cells, both *in vitro* and *in vivo* (Fig. 3B,C).

***In vivo* transplantation of tumor cells.** When the HEC1A/LUC cells were injected into the uterus, subsequent tumor development was observed; therefore, follow ups were performed to determine the occurrence of lymph node metastases (Table 1). At 4 weeks, one of five mice developed metastasis; at 6 weeks, two of four developed metastasis. At 8 weeks after injection, all of the mice in the first series exhibited lymph node metastasis. Consequently, the second series was carried out using a larger number of animals, which again resulted in the successful development of metastases within the same time frame. At 8 weeks, the uterus was swollen and the endometrium was filled with tumor cells (Fig. 4A,B). Marked infiltrations into the enlarged lymph nodes were also noted in mice injected with the HEC1A/LUC (Fig. 4C) and HEC1A/VEGF-C (Fig. 4D,E) cells.

Relationship between lymph node size and metastasis. Upon analysis of the model mice, 40 lymph nodes were selected by size and excised for histological evaluation. Lymph nodes larger than 3 mm in longitudinal diameter were all positive for tumor metastasis, whereas smaller ones exhibited lower positivity (Fig. 5A).

Effect of VEGF-C on lymph node metastasis. The results of the third and fourth series revealed a significant increase in the number of metastases when VEGF-C was overexpressed by the cells (1.1 ± 0.8 *vs* 3.2 ± 1.3, third and fourth series, respectively). Statistical significance was determined by the Mann-Whitney *U*-test (Fig. 5B).

Reproducibility of lymph node metastasis. As shown in Table 1, the majority of animals developed lymph node metastasis at 8 weeks after injection. The overall rate of positive lymph node metastasis was 86.5% (45/52 animals). All lymph nodes >3 mm in longitudinal diameter contained tumor cells, as determined by histological analysis (Fig. 5B). No animals exhibited direct invasion of the tumor or metastatic lesions other than the lymph nodes.

Lymphangiogenesis in the subcutaneous tumor. After 2 weeks of subcutaneous inoculation, the tumors were analyzed histochemically. The tumor tissue based on the HEC1A/VEGF-C cells exhibited a marked increase in VEGFR3-positive vessels compared with the HEC1A/LUC cells (Fig. 6A,B). The number

Table 1. Number of animals with lymph node metastasis

Weeks	4	6	8
First series (HEC1A/LUC)	1/5	2/4	4/4
Second series (HEC1A/LUC)	–	–	8/8
Third series (HEC1A/LUC)	–	–	15/21
Fourth series (HEC1A/VEGF-C)	–	–	18/19

HEC1A, an endometrial cancer cell line; LUC, luciferase; VEGF-C, vascular endothelial growth factor-C.

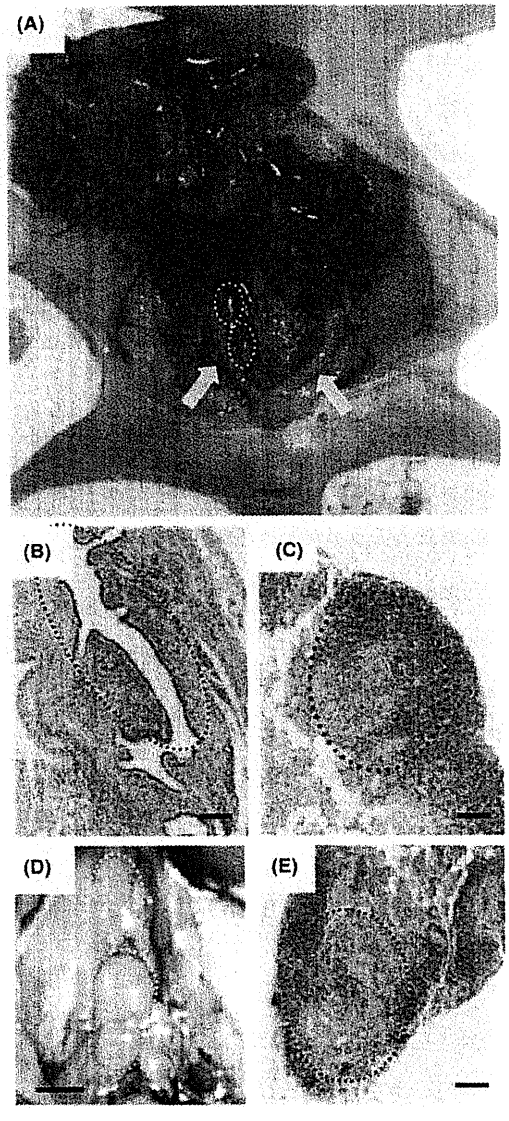

Fig. 4. Involvement of cancer cells *in vivo*, 8 weeks after injection. (A–C) animals were injected with HEC1A/luciferase cells. (A) Enlarged uterus (arrows) and lymph nodes (dotted circles) are shown. No other metastatic sites were found in these animals. (B) Micrograph of a uterus. Tumor cells in the endometrial zone, which resemble a uterus with endometrial cancer. (C) Enlarged lymph node with tumor metastasis. (D) Para-aortic lymph node swelling was observed in mice injected with HEC1A/vascular endothelial growth factor-C cells. Dotted circles indicate enlarged lymph nodes. (E) Lymph node metastasis was confirmed by histological evidence of tumor infiltration. Bars in (B), (C), and (E) indicate 1 mm; bar in (D) indicates 4 mm.

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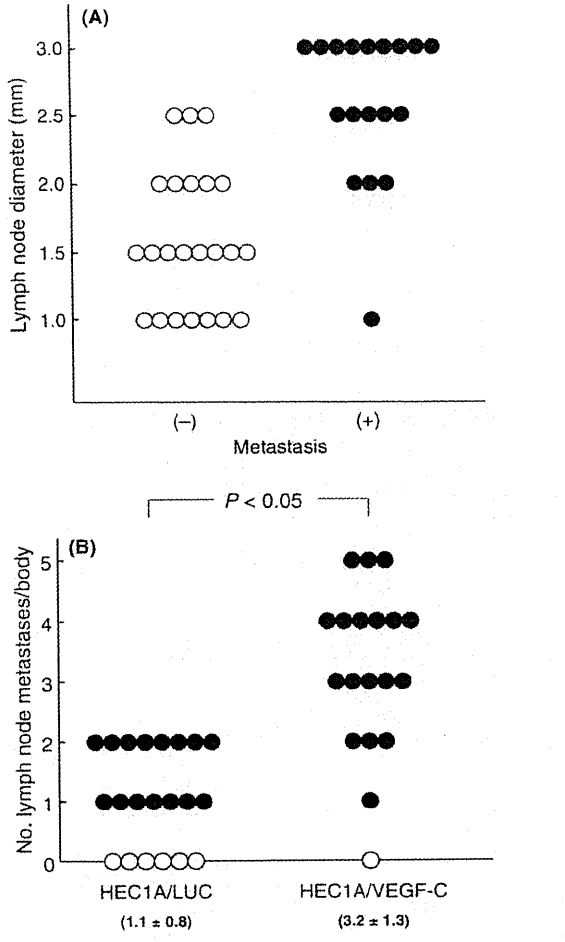

Fig. 5. Number of lymph nodes in animals, 8 weeks after tumor injection. (A) Relationship between lymph node size (longitudinal diameter) and metastasis is shown. Closed circles indicate histologically-positive metastasis. (B) Number of metastatic lymph nodes larger than 3 mm in longitudinal diameter at 8 weeks. Results from the third and fourth series are shown. Closed circles indicate histologically-positive metastasis. Animals injected with HEC1A/vascular endothelial growth factor-C (VEGF-C) showed a higher number of lymph node metastases. LUC, luciferase.

of VEGFR3-positive vessels in each group ($n = 4$) were counted using microscopy, and the results were analyzed. A significant increase was observed in tumors originated from HEC1A/VEGF-C cells (Student's t-test, Fig. 6C).

Discussion

In the present study, we developed a unique lymph node metastasis model by orthotopically injecting endometrial cancer cells. In this method, most of the animals exhibited lymph node metastasis, along with uterine involvement in cancer. The reproducibility of the metastases seen in our experiment is extremely

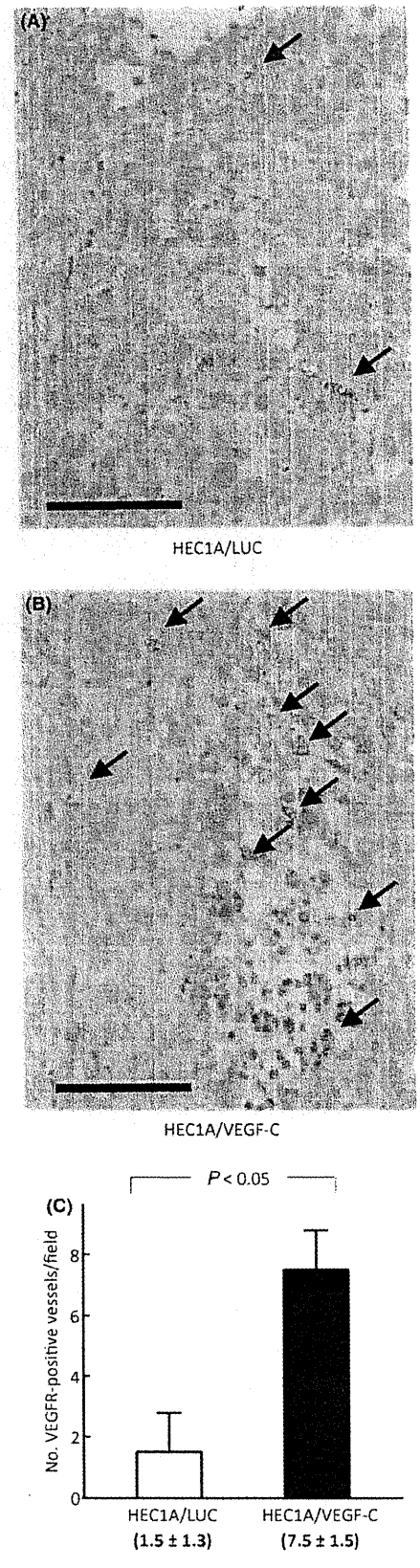

Fig. 6. Immunohistochemical analysis of subcutaneously-inoculated tumor. Arrows indicate vascular endothelial growth factor (VEGF) receptor 3 (VEGFR3)-positive vessels, possibly reflecting lymphangiogenesis in HEC1A/luciferase (LUC) (A) and HEC1A/VEGF-C cells (B). Bars indicate 100 μm. (C) Number of vessels positive for VEGFR3 was enumerated under a light microscope at ×20 magnification. Four animals were used for each cell.

important in developing a model for lymph node metastasis. In our model, all animals survived the injection procedure, and 45 of 52 mice (86.5%) exhibited positive lymph node metastasis. This rate is remarkably high, thus making this model reliable for further therapeutic interventions. In all of the animals, uterine tumor growth was local and self-limited, and the peritoneal dissemination of other sites of metastasis was not evident. These features considerably reflect the clinical conditions of lymph node metastasis, assuring the utility of the model.

Nonetheless, when the present pathological findings are compared to those of human cases, there are slight differences in the metastatic regions. In the mice used in the present experiments, only para-aortic lymph node areas were involved, whereas in humans, pelvic lymph nodes are also metastasized. While this difference might be due to differences in lymphatic anatomy, it would be advantageous for researchers to evaluate metastatic conditions more clearly.

There are a number of decisions to be made when developing models, such as choosing cell lines, the number of cells for injection, various technical details, and observation periods. We initially selected HEC1A cells, because they are one of the most widely used endometrial cancer cell lines. Based on our experience, HEC1A cells appeared to be the most suitable for the present study; lymph node metastasis was well developed before other potential metastatic sites or original growths. One of the most important aspects is the invasiveness of the cells. HEC1A cells are not very invasive by nature, and did not cause peritoneal dissemination in our series of experiments during the observation period. In fact, we also tested HEC50B cells and found that although metastatic lesions were found earlier (by 4–5 weeks), tumor invasion into the surrounding areas was prominent, and consequently, the animals were not suitable for further evaluation of metastasis. The invasiveness seems unrelated to the levels of VEGF-C production, but related to the state of differentiation. HEC1A cells are known to be well differentiated, whereas HEC50B cells are poorly differentiated. In this respect, Ishikawa (3H12) cells might be a good candidate, as they are classified as well-differentiated carcinoma; nonetheless, they are also known to transform into undifferentiated status under prolonged culture.[11] For this reason, we did not test this cell line for the development of the model.

The number of cells per injection is another important factor when developing a model. As a general rule, the more cells that are injected, the earlier the disease develops. The number of cells used in this study (5×10^6) was near maximum, because the least amount of solution to suspend the cells is close to the maximum volume for intrauterine injections in BALB/c mice (approximately 80 μL). It is also imperative to keep the tumor cells within the uterine cavity after injection. To do so, it is important that the distal ends of the uterus and cervix are appropriately ligated, while avoiding excessive amounts of injection. If the cells flow out from the distal end of the uterus, they might spread into the peritoneal space, leading to intraperitoneal tumor dissemination. Alternatively, if the cells are lost through the vagina, the inoculation is deemed unsuccessful. In our experiments, no significant intraperitoneal regions were observed in the animals, even when lymph node metastases were not evident.

Two previous studies established lymph node metastasis models by injecting cancer cells into the uterus. The first study utilized the metastatic subline PL3 of rat Walker 256 cells.[15] This cell line originates from mammary tumor cells, and the metastatic subline was enriched after more than five cycles of *in vivo* selection. Another study orthotopically implanted MH and KF cells; both originate from human ovarian cancer.[16] The latter study also established highly metastatic sublines (MH–LN3 and KF–LN3) after three cycles of *in vivo* selection. Therefore, the *in vivo* selection of the cells is essential before establishing a metastasis model. Meanwhile, the stability of the metastatic sublines, along with their availability, is not clear. In this study, we utilized publicly-available cell lines without *in vivo* selection steps. These features, along with the high rate of reproducibility, make this model particularly useful for evaluating lymph node metastasis.

At the time of histological evaluation, various sizes of lymph nodes were found, and it was difficult to estimate whether they were metastatic or not. To simplify the evaluation steps, we tested for the presence of metastatic tumors in lymph nodes by size. As shown in Figure 5(A), all lymph nodes larger than 3 mm were positive for metastasis. Therefore, we enumerated lymph nodes by using this size limit. In addition, the lymph nodes shown in Figure 5(B) were also analyzed; the additional 84 lymph nodes were all positive for metastasis. With these results, we are confident that we can estimate the metastasis solely by the size of the lymph node, at least in this model.

Based on our results, it is clear that the overexpression of VEGF-C results in an enhancement of lymph node metastasis (Fig. 5B). The use of this particular cell line results in a more robust model for lymph node metastasis. It is very likely that the activity of VEGF-C secreted from the tumors facilitates lymph node metastasis. In the present study, we demonstrated significant increase of VEGFR3-positive vessels within the tumor, suggesting enhanced lymphangiogenesis by VEGF-C (Fig. 6). The precise mechanism of VEGF-C, with regard to lymph node metastasis, is not well understood; few existing studies focus on this point. One study suggests that lymphatic endothelial cell (LEC) migration, rather than proliferation, is responsible for metastasis in pancreatic cell lines.[17] In that study, the relationship between VEGF-C concentration and the number of migrating LEC exhibited a positive but non-linear correlation; this is quite similar to our observation between VEGF-C and the number of lymph node metastases. In any case, it is expected that blocking the activity of VEGF-C would suppress lymph node metastasis. We are currently preparing a therapeutic experiment that incorporates soluble VEGFR3 into this model.

The prognosis of endometrial carcinoma with lymph node metastasis is poor, and few improvements have been made. The present model might offer a platform on which therapeutic progress against lymph node metastasis can be made.

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Disclosure Statement

The authors declare no financial or commercial conflict of interest.

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Letter to the Editor

Free school-based vaccination with HPV vaccine in a Japanese city

Keywords:
Human papillomavirus
Cervical cancer
School-based vaccination

In early 2000, the human papillomavirus (HPV) vaccine was developed to prevent cervical cancer. Quadrivalent and bivalent (AS04-adjuvanted) HPV vaccines were approved in 2006 and 2007, respectively, in Australia, the European Union, and the United States, among others. Large-scale clinical trials have shown that quadrivalent [1] and bivalent HPV vaccines [2,3] effectively prevent the development of cervical intraepithelial neoplasia (CIN)2 and CIN3. Three years behind the European Union, the bivalent HPV AS04-adjuvanted vaccine was approved in October 2009 for use in Japan.

To eliminate cervical cancer using the HPV vaccine, a high rate of immunisation with this vaccine must be achieved. A prerequisite for increasing the rate of immunisation is public financial support for the cost of vaccination. In October 2009, such support has not yet been established at the national level for this vaccine in Japan. A second requirement for achieving a greater rate of immunisation is ensuring the availability of vaccination. Group vaccinations at grade schools and middle schools would offer a particularly effective method of increasing the rate of immunisation [4,5]. The Japanese Preventive Vaccination Act was revised in 1994, requiring all vaccinations to be accomplished by private vaccinations rather than by school-based group vaccinations.

Ohtawara City is a small city with a population of approximately 80,000, located about 130 km (80 miles) northeast of Tokyo. Ohtawara City recognised that vaccination with the HPV vaccines was essential to controlling the development of cervical cancer. In February 2010, the city decided to fully subsidise the costs of vaccinations for all 12-year-old (6th grade) girls residing in Ohtawara City, and established school-based group vaccinations to achieve higher rates of immunisation. In May 2010, the first group vaccinations of the HPV vaccines in Japan began in all the grade schools of Ohtawara City.

A total of 340 pupils were targeted for vaccination in Ohtawara City, of whom 336 were willing to be vaccinated (98.8%). A total of 315 pupils (92.6% of total targeted pupils; 93.8% of those willing to be vaccinated) received their first vaccination in grade schools. Pupils who could not be vaccinated because they were unwell (e.g., fever) on the appointed day were vaccinated later at school ($n = 5$) or by their doctor ($n = 15$), for a final total of 335 vaccinated pupils (98.5% of total targeted pupils; 99.7% of those willing to be vaccinated). The second and third vaccinations were similarly given, as shown in Table 1, and extremely high rates of immunisation have been achieved. No serious complications such as anaphylactic shock have been encountered.

The requirements for achieving a high rate of immunisation with HPV vaccines include: (1) public subsidies to cover the costs of vaccination; (2) wide availability, such as school-based vaccination; and (3) awareness and education regarding HPV vaccines and cervical cancer. An extremely high rate of immunisation was achieved in Ohtawara City through full subsidisation of vaccination costs and school-based group vaccination. Implementing educational programs for parents and children is also important. The health promotion section of the Ohtawara City government invited a medical specialist and held multiple open lectures regarding cervical cancer and HPV vaccine specifically for parents and teachers. We also created leaflets containing medical information about this program, and school teachers educated children using these leaflets. For foreign students, leaflets written in English were created and distributed. Ohtawara City also enjoys a wealth of educational activities involving healthcare professionals from health outreach centres for a variety of diseases such as cancer, and has a heightened awareness of public health issues. Municipal authorities also enthusiastically approached city medical associations, city grade school principals, healthcare teachers, and the like in their efforts regarding vaccination with HPV vaccines, and successfully built a fully prepared coalition. Presentations on cervical cancer and the HPV vaccines were also held for the public in order to improve public awareness. All of this was also a factor in achieving a high rate of immunisation. In addition, group vaccinations with the HPV vaccines were entirely voluntary, not compulsory, and were started with "gentle encouragement".

Prompted by these efforts in Ohtawara City, free school-based vaccinations have been established in four more cities and towns in Tochigi Prefecture, where the city of Ohtawara is located. Although data are only available for the first and second vaccinations, the mean rate of immunisation in these four cities has been over 90%, as in Ohtawara City. The Ministry of Health, Labour and Welfare has implemented a requirement for parents to attend vaccinations in elementary school from 2011. In Tochigi Prefecture, the school-based vaccination for sixth grade elementary school students was changed to individual vaccinations. The school-based vaccination for junior high school students is scheduled to continue as long as finances permit.

Table 1
Number of vaccines provided to targeted pupils.

	n	%
Number of pupils targeted	340	-
Number willing to be vaccinated	336	98.8
Number of vaccinations (school/individual)		
1st	335 (315/20)	98.5
2nd	335 (318/17)	98.5
3rd	328 (307/21)	96.5

%: relative to the number of pupils targeted.

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