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ORIGINAL PAPER

Usefulness of third-line chemotherapy for women with recurrent ovarian, fallopian tube, and primary peritoneal cancer who receive platinum/taxane regimens as first-line therapy

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Abstract
Background Limited information is available regarding the usefulness of third-line chemotherapy for recurrent ovarian, fallopian tube, and primary peritoneal cancer treated with platinum-taxane regimens as first-line therapy.
Patients and methods We retrospectively reviewed the medical records of women with ovarian, fallopian tube, and primary peritoneal cancer who were treated at the National Cancer Center Hospital between 1999 and 2005 to investigate the relations of clinicopathological factors to important clinical endpoints such as the response rate (RR), time to progression (TTP) and overall survival (OS) after third-line chemotherapy.
Results A total of 172 patients received first-line platinum/taxane regimens during the study period, among whom 111 had disease progression after first-line chemotherapy. Eighty-one of these 111 patients received second-line chemotherapy, and 73 had disease progression. Fifty-four of the 73 patients with disease progression received third-line chemotherapy. The RR to third-line chemotherapy was 40.7% (95% CI, 27.6–53.8%). The median TTP was 4.4 months (range 0–19.5 months), and the median OS was 10.4 months (range 1.5–44.3 months). Performance status (PS) and primary drug-free interval (DFI) were independent predictive factors for the RR to third-line chemotherapy ($P = 0.04$ and $P = 0.009$). PS and primary DFI were also independent predictive factors for TTP and OS on multivariate analysis ($P = 0.006$, $P = 0.005$ and $P = 0.01$, $P = 0.004$, respectively).
Conclusions PS and primary DFI are useful predictors of the response to third-line chemotherapy in women with recurrent ovarian, fallopian tube, and primary peritoneal cancer. In this setting, however, both of these variables are subject to several well-established potential biases and limitations; further prospective studies are thus needed.

Keywords Third-line chemotherapy · Recurrent ovarian · Fallopian tube · Primary peritoneal cancer

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Introduction

Ovarian cancer remains the leading cause of death from gynecological neoplasms in the Western world (Greenlee et al. 2001). Most cases are diagnosed when the disease is advanced, resulting in poor survival (Heintz et al. 2001; Engel et al. 2002; Jemal et al. 2003). Despite high rates of objective responses to surgery and primary chemotherapy, relapse rates remain high. Recurrent disease is treated either with the same regimen as that used for first-line chemotherapy (i.e., reinduction therapy) or with second- or third-line regimens. The aim of treatment after relapse is mainly palliative, designed to control disease symptoms, maintain patients' quality of life, and prolong survival. New chemotherapeutic drugs have yielded objective response rates

(RR) of 10–30% in recurrent ovarian cancer, depending on the anticancer activity of the drug(s) used, cross-resistance with previously administered drugs, and the response of the primary tumor to platinum compounds. In general, primary platinum sensitivity is defined as a documented response to the initial platinum-based therapy for at least 6 months after the end of treatment. RR of 30% to >50% have been obtained in patients with longer treatment-free intervals or with platinum-sensitive primary tumors (primary platinum sensitivity) as compared with only <20% in patients with shorter treatment-free intervals (platinum resistance of the primary tumor) or refractory ovarian cancer (no remission in response to first-line therapy) (Blackledge et al. 1989; Markman et al. 1991; Thigpen et al. 1994). Most previous studies have focused on the overall tumor response and time to treatment failure for specific drugs rather than attempting to evaluate the overall response to second-, third-, or fourth-line chemotherapy. The aim of this retrospective study was to investigate the relations of clinicopathological factors to important clinical endpoints such as the RR, time to progression (TTP), and overall survival (OS) in response to third-line chemotherapy in women with recurrent ovarian, fallopian tube, and primary peritoneal cancer who received platinum/taxane regimens as first-line therapy.

Patients and methods

Patients

We retrospectively reviewed the medical records of patients with ovarian, fallopian tube, and primary peritoneal cancer treated at the National Cancer Center Hospital between 1999 and 2005. All the patients had received platinum/taxane regimens as first-line therapy. Treatment decisions were usually made by the attending clinician. Patients in whom the tumor was considered possibly platinum-sensitive usually received a platinum agent, a taxane, or both. In general, combination chemotherapy was not administered as salvage treatment for recurrent disease, and most patients with recurrent disease received a single chemotherapeutic agent. Drug-free interval (DFI) was measured from the date of the last dose of chemotherapy until disease progression. Primary DFI was measured from the date of last dose of first-line chemotherapy until disease progression, and secondary DFI was measured from the date of the last dose of second-line chemotherapy until disease progression. Patients participated in clinical trials if they were eligible. The imaging criteria for treatment response were based on two-dimensional measurements of the lesions. Serum CA125 levels were not used as a primary measure of the response, but were referred to in the evaluation of response. Complete response was defined as no evidence of disease on physical examination or imaging studies, with normalization of the serum CA125 level. Partial response was defined as a >50% reduction in tumor size. Stable disease was defined as a 25–50% decrease or increase, or as no change in tumor size. Patients with an increase in the serum CA125 level were not evaluated to have had a partial response or stable disease. Progressive disease was defined as a >25% increase in tumor size. The Response Evaluation Criteria in Solid Tumors (RECIST) criteria were not used because most patients received treatment before this system was adopted by our hospital.

Statistical analysis

The main outcome measures for drug efficacy were RR, TTP, and OS. TTP was defined as the interval from the first day of third-line chemotherapy to the day of documented disease progression. For patients who were alive at the end of the study, the TTP data were right-censored to the time of the last evaluation or the time of the last contact at which the patient was progression-free. OS was defined as the interval from the first day of third-line chemotherapy to the day of death. For patients who were alive at the end of the study, the OS data were right-censored to the time of the last evaluation or contact. Data were analyzed by parametric and nonparametric statistics using SAS, version 9.1.3 (SAS Institute, Cary, NC, USA). Descriptive statistics were used for demographic data; such data are presented as mean with standard deviations or as medians with ranges. Survival was estimated using the Kaplan–Meier method, and differences between survival curves were evaluated with the log-rank test. A multivariate logistic regression analysis was performed to determine predictive factors of the response to chemotherapy. A Cox regression analysis was performed to determine factors influencing TTP and OS.

Results

A total of 172 patients received first-line platinum/taxane regimens during the study period, of whom 111 had disease progression after first-line chemotherapy. Eighty-one of these 111 patients received second-line chemotherapy, among whom 73 had disease progression. Fifty-four of these 73 patients received third-line chemotherapy (Fig. 1). Mean age at the time of diagnosis of the primary cancer was 54 years (26–75 years), and mean age at the start of second- and third-line chemotherapy was 55 (28–76 years) and 55 (31–77 years) years, respectively. There were 46 cases (85.1%) of ovarian carcinoma, 7 (13.1%) of primary peritoneal carcinoma, and 1 (1.8%) of fallopian tube carcinoma. The patients' characteristics are shown in Table 1.

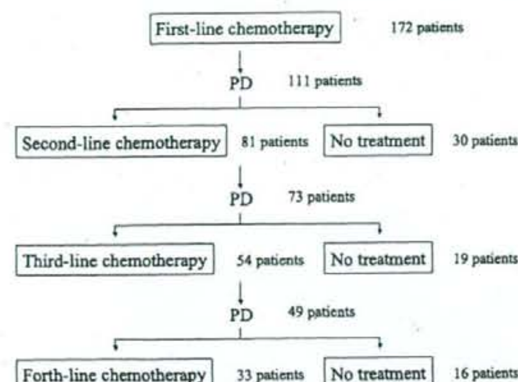

Fig. 1 Schema of treatment

At the time of initial recurrence, 37 patients (69%) had primary platinum sensitivity, and 17 (31%) had primary platinum resistance. All patients treated with second-line drugs had either stable or progressive disease and received third-line treatment within 1–2 months after the last cycle of second-line treatment. The most commonly used regimen was weekly paclitaxel/carboplatin for second-line treatment and carboplatin for third-line treatment. The numbers of patients and chemotherapeutic drugs used in each setting are listed in Table 2. The median number of cycles of third-line treatment was 6 (range 1–18 cycles). The median TTP was 4.4 months (range 0–19.5 months), and the median OS was 10.4 months (range 1.5–44.3 months) (Fig. 2). The RR to third-line chemotherapy was 40.7% (95% CI; 27.6–53.8%). Five patients (9.2%) had complete responses and 17 (31.5%) had partial responses. Disease remained stable in 18 patients (33.3%) and progressed in 12 (22.2%). Two patients discontinued treatment because of hypersensitivity reactions to carboplatin. Overall, 49 patients had disease progression and 33 subsequently received fourth-line chemotherapy. The RR to fourth-line chemotherapy was 36.3% (95% CI, 19.9–52.7%); 2 patients had complete responses, 7 had partial responses, 9 had stable disease, and 12 had progressive disease. At the time of data analysis, 38 of the 54 patients (70.3%) had died. We studied the relations between the response to third-line drug therapy and clinical factors such as age, performance status (PS), histopathological type of cancer, number of target lesions, primary and secondary DFI, response to second-line chemotherapy, and the use of platinum/taxane regimens. The RR to third-line treatment was found to be significantly better in patients with a good PS (0 or 1) and a primary DFI of >6 months ($P = 0.04$ and $P = 0.009$, respectively, Table 3). Patients with a good PS and a primary DFI of >6 months also had a longer TTP and better OS ($P = 0.006$, $P = 0.005$ and $P = 0.01$, $P = 0.004$, respectively, Table 4). Median OS was slightly but not significantly better in the patients who responded to third-line chemotherapy (15.1 months; range 2.4–33.7 months) than in those who did not (9.4 months; range 1.5–44.3 months) ($P = 0.054$, Fig. 3). Median OS was significantly longer in the patients who received fourth-line chemotherapy (8.2 months; range 2.1–25.2 months) than in those who did not receive chemotherapy (2.4 months; range 0.2–16.2 months) ($P < 0.0001$, Fig. 4).

Table 1 Patient characteristics ($n = 54$)

Age (year)	Median (range)
At primary diagnosis	54 (26–76)
At second-line chemotherapy	55 (28–77)
At third-line chemotherapy	55 (31–78)
Performance status	
0	6
1	22
2	24
3	2
Stage	
I	4
II	5
III	30
IV	15
Organ	
Ovarian carcinoma	46
Primary peritoneal carcinoma	7
Fallopian tube carcinoma	1
Pathology	
Serous adenocarcinoma	40
Endometrioid adenocarcinoma	2
Mucinous adenocarcinoma	1
Clear cell carcinoma	5
Undifferentiated carcinoma	6
No. of target lesions	
1	38
2	10
3	6
Drug free-interval (month)	
Primary	8.2 (0.9–39.3)
Secondary	8.3 (0.1–21.5)
3rd-line regimens	
Platinum/taxane-containing regimens	36
Other regimens	18

Discussion

In women with ovarian cancer, treatment goals after failure to respond to first-line therapy are (1) the control or prevention

Table 2 First-, second- and third- line chemotherapeutic regimens used

First-line		Second-line		Third-line	
Paclitaxel/carboplatin	35	Weekly paclitaxel/carboplatin	28	Carboplatin	14
Docetaxel/carboplatin	12	Docetaxel/carboplatin	12	Weekly paclitaxel/carboplatin	10
Paclitaxel/cisplatin	7	Irinotecan	5	Irinotecan	8
		Topotecan	2	Irinotecan/etoposide	4
		Carboplatin	2	Docetaxel	4
		Liposomal doxorubicin	1	Liposomal doxorubicin	4
		Paclitaxel/carboplatin	1	Docetaxel/carboplatin	3
		Irinotecan/carboplatin	1	Cisplatin	2
		Irinotecan/etoposide	1	Paclitaxel/carboplatin	1
		Docetaxel	1	Paclitaxel/cisplatin	1
				Irinotecan/mitomycin	1
				Paclitaxel	1
				Etoposide	1

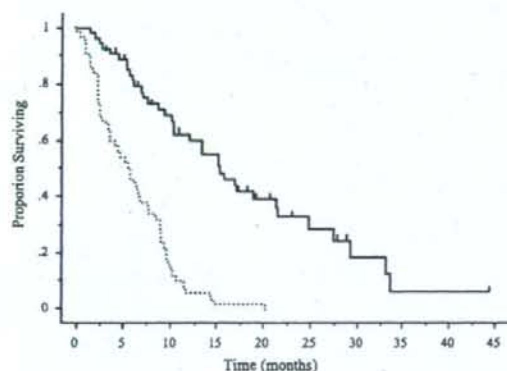

Fig. 2 Kaplan–Meier analysis of time to progression (*solid line*) and overall survival (*dotted line*) following third-line chemotherapy. *Vertical bars* indicate censored cases

of disease-related symptoms, (2) the maintenance of a good quality of life, and (3) the prolongation of progression-free survival. The aims of salvage treatment have long been a matter of debate. The possibility of achieving an OS benefit in these patients is very limited. RRs are generally similar to or poorer than those with previous treatments are. Moreover, the increased risk of toxicity in patients with a history of previous treatment(s) and of negatively affecting performance status makes some physicians reluctant to continue drug treatment.

Patients who have good performance status without clinically significant comorbidity may wish to continue treatment (Doyle et al. 2001). Donovan et al. (2002) evaluated the treatment preferences of women with recurrent ovarian cancer and reported that most patients (86%) initially prefer subsequent therapy, with 25% never considering the withdrawal of chemotherapy, even when the expected median survival was <1 week. Physicians must therefore take into account patients' wishes along with other clinical data when planning treatment.

Most studies of salvage therapy have focused on the response to a particular single- or combined-drug regimen.

Table 3 Multivariate analysis of response rates to third-line chemotherapy

Clinical factors	No. of patients	Response rate (95% CI)	*P* value
Age			
<60	35	52.6% (30.1–75.0%)	0.50
≥60	19	44.0% (23.7–56.2%)	
PS			
0.1	28	57.1% (38.8–75.4%)	0.04
2.3	26	30.7% (13.0–48.5%)	
Pathology			
Mucinous/clear cell	6	33.3% (4.3–71.0%)	0.22
Non-mucinous/clear cell	48	45.8% (31.7–59.9%)	
No. of target lesions			
1	40	45.0% (29.5–60.4%)	0.37
2.3	14	42.8% (16.9–68.7%)	
Primary DFI			
<6 months	17	17.6% (0.4–35.7%)	0.009
≥6 months	37	51.3% (35.2–67.4%)	
Secondary DFI			
<6 months	33	42.4% (25.5–59.2%)	0.70
≥6 months	21	47.6% (26.2–68.9%)	
Response to second-line therapy			
Responders	35	45.7% (29.2–62.2%)	0.09
Non-responders	19	31.5% (10.6–52.4%)	
PT regimens			
PT regimens	36	38.8% (22.9–54.8%)	0.75
Non-PT regimens	18	44.4% (21.4–67.4%)	

PT Platinum/taxane

Table 4 Multivariate analysis of TTP and OS following third-line chemotherapy

Clinical factors	TTP *P* value	OS *P* value
Age (<60 vs. ≥60)	0.59	0.76
PS (0.1 vs. 2.3)	0.006	0.005
Pathology (mucinous/clear cell vs. non-mucinous/clear cell)	0.34	0.29
No. of target lesions (1 vs. 2.3)	0.85	0.79
Primary DFI (<6 months vs. ≥6 months)	0.01	0.004
Secondary DFI (<6 months vs. ≥ 6 months)	0.61	0.34
Response to second-line therapy (responder vs. non-responder)	0.43	0.17
PT regimens (PT regimens vs. non-PT regimens)	0.84	0.36

DFI Drug free-interval, *PT* Platinum/taxane

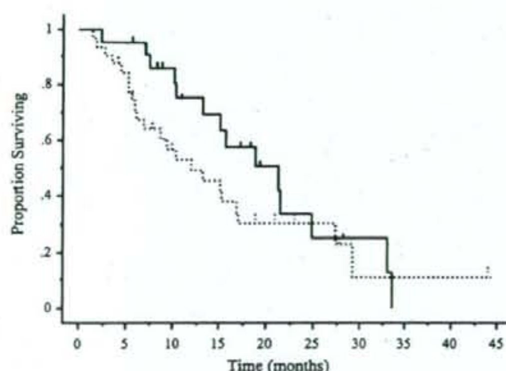

Fig. 3 Kaplan–Meier analysis of overall survival (*bottom*) following third-line chemotherapy. The difference between third-line responders (*solid line*) and third-line non-responders (*dotted line*) was not statistically significant ($P = 0.054$). *Vertical bars* indicate censored cases

Without well-designed controlled studies, however, it is difficult to determine outcomes that would be obtained if a drug were used earlier or later in the course of salvage treatment.

In our study, the RR to third-line chemotherapy was 40.7% (95% CI; 27.6–53.8%). To date, only a few authors have distinguished between second- and third-line treatments when evaluating drug response and survival rates. In a study by Villa et al. (1999), 49 patients with recurrent ovarian cancer received third-line drugs after complete or partial responses to second-line chemotherapy. The overall RR was 48%, and median survival was 6 months. The 1-year survival rate differed significantly between patients who responded and those who did not respond to second-line treatment (82 vs. 39%, $P < 0.05$).

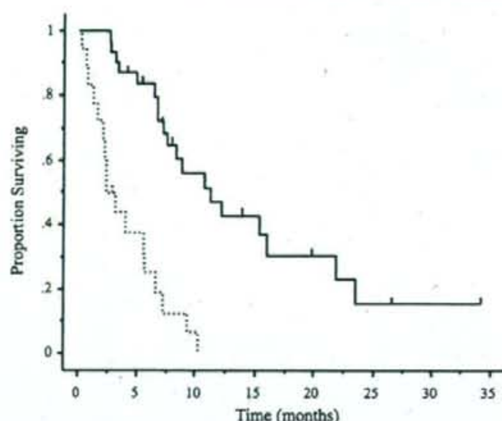

Fig. 4 Kaplan–Meier analysis of overall survival following fourth-line chemotherapy. The chemotherapy group (*solid line*) had significantly better survival ($P < 0.0001$) than the non-chemotherapy group (*dotted line*). *Vertical bars* indicate censored cases

We obtained good RRs to third-line treatment in patients with a good PS and those with a primary DFI of >6 months. The response to third-line chemotherapy is influenced by the response to second-line chemotherapy. However, third-line chemotherapy has only a modest RR with a marginal prolongation of progression-free interval, but no obvious effect on survival in patients with ovarian cancer (Tangjitgamol et al. 2004).

In our study, there was no significant difference in the survival rate between patients responded to third-line chemotherapy and those who did not. In terms of cost-effectiveness, best supportive care is the only cost-effective strategy, followed perhaps by second-line monotherapy, given currently available chemotherapeutic options (Rocconi et al. 2006).

Patients with recurrent disease are often retreated with the same primary drug(s), most often platinum agents, but might also receive other drugs (Markman et al. 1991; Thigpen et al. 1993; Bookman 2003; Fung et al. 2002). One of the most important considerations in selecting second-line therapy is platinum sensitivity status, as defined by the response of the primary disease to a platinum drug and the progression-free interval after the completion of treatment (Blackledge et al. 1989; Thigpen et al. 1993; Thigpen et al. 1994). Some researchers have argued that there is no definite treatment-free interval, which can reliably distinguish platinum sensitivity from platinum resistance (Markman 1998; Markman et al. 1998). Nonetheless, it is generally accepted that the longer the treatment-free interval, the better is the expected response to retreatment (Markman et al. 1991; Thigpen et al. 1994). One study reported that women with recurrent ovarian cancer who had a treatment-free

interval of between 5 and 12 months showed a RR of only 27% to second-line platinum-based therapy, as compared with 59% in those with a treatment-free interval of longer than 24 months (Markman et al. 1991). However, the duration of secondary response to platinum therapy is less well documented; in particular, the relation between the duration of secondary response and that of the initial response is poorly understood.

Our results showed that PS and primary DFI were useful predictors of the response to third-line chemotherapy. Eisenhauer et al. (1997) conducted a multivariate analysis to determine predictors of clinical response to subsequent chemotherapy in 704 patients with platinum-pretreated ovarian cancer. Their initial univariate analysis revealed that response was significantly associated with many factors, including the drug used, time since diagnosis, tumor size, histology, and the presence or absence of liver metastasis. In contrast, their multivariate analysis showed that only serous histologic type, number of disease sites ≤ 2, and maximum size of the largest lesion <5 cm were associated with a favorable response. They concluded that drug activity might not be the only determinant of response, and that tumor characteristics are also important factors.

In our study, two patients had hypersensitivity reactions to carboplatin. Patients who receive multiple courses of carboplatin have increased rates of hypersensitivity reactions (Zanotti et al. 2001). The incidence of such reactions is 27% in patients receiving 7 or more cycles of carboplatin, with more moderate to severe symptoms developing in more than 50% of these patients (Markman et al. 1999).

The time to treatment failure (4.4 months) and the OS (10.4 months) in our study were consistent with those reported by other studies assessing individual drugs (Villa et al. 1999; Heintz et al. 2001; Tangjitgamol et al. 2004; Rocconi et al. 2006). The survival of patients given fourth-line and subsequent treatment was significantly longer than that of patients who received no further therapy after third-line treatment (8.3 months vs. 2.4 months, respectively; $P < 0.0001$). Administration of fourth-line chemotherapy to patients who might tolerate such treatment may also improve OS; however, the analysis of OS in this setting has its limitations and is prone to potential bias. One study reported that giving additional lines of chemotherapy may not improve OS and that the inclusion of paclitaxel in treatment regimens may have a significant effect on survival (Findley et al. 2005).

In conclusion, our study suggested that PS and primary DFI may be useful predictors of the response to third-line chemotherapy in women with recurrent ovarian, fallopian tube, and primary peritoneal cancer. Our findings will hopefully help physicians make treatment recommendations and inform patients about expected benefits and risks, outcomes, and survival rates in this setting. Finally, the decision whether to use third-line chemotherapy should be based on a comprehensive assessment of patients' wishes, drug efficacy and toxicity, and treatment expertise of the clinician.

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Frequent Inactivation of a Putative Tumor Suppressor, Angiopoietin-Like Protein 2, in Ovarian Cancer

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Abstract

Angiopoietin-like protein 2 (ANGPTL2) is a secreted protein belonging to the angiopoietin family, the members of which are implicated in various biological processes, although its receptor remains unknown. We identified a homozygous loss of *ANGPTL2* (9q33.3) in the course of screening a panel of ovarian cancer (OC) cell lines for genomic copy-number aberrations using in-house array-based comparative genomic hybridization. *ANGPTL2* mRNA expression was observed in normal ovarian tissue and immortalized normal ovarian epithelial cells, but was reduced in some OC lines without its homozygous deletion (18 of 23 lines) and restored after treatment with 5-aza 2′-deoxycytidine. The methylation status of sequences around the *ANGPTL2* CpG-island with clear promoter activity inversely correlated with expression. *ANGPTL2* methylation was frequently observed in primary OC tissues as well. In an immunohistochemical analysis of primary OCs, ANGPTL2 expression was frequently reduced (51 of 100 cases), and inversely correlated with methylation status. Patients with OC showing reduced ANGPTL2 immunoreactivity had significantly worse survival in the earlier stages (stages I and II), but better survival in advanced stages (stages III and IV). The restoration of ANGPTL2 expression or treatment with conditioned medium containing ANGPTL2 inhibited the growth of OC cells originally lacking the expression of this gene, whereas the knockdown of endogenous ANGPTL2 accelerated the growth of OC cells with the expression of ANGPTL2. These results suggest that, at least partly, epigenetic silencing by hypermethylation of the *ANGPTL2* promoter leads to a loss of ANGPTL2 function, which may be a factor in the carcinogenesis of OC in a stage-dependent manner. [Cancer Res 2008;68(13):5067–75]

Introduction

Epithelial ovarian cancer (OC) is the most common and lethal gynecologic malignancy and is one of the leading causes of cancer mortality in women because the disease usually presents at an advanced stage, as there are no overt symptoms at early stages (1, 2). Despite the use of primary surgical cytoreduction and systemic administration of paclitaxel-containing and platinum-containing chemotherapy regimens, minimal improvements have been made in overall survival over the past three decades. Therefore, a critical need exists for the identification of molecular markers and targets for diagnosis as well as therapy, which will come from a better understanding of the molecular mechanisms responsible for the tumorigenesis of this disease (3).

Sporadic OCs often show complex, aneuploid karyotypes, with a myriad of nonrandom structural chromosomal abnormalities (4), which may activate oncogenes or inactivate tumor suppressor genes (TSG) during the transformation process. To identify novel candidates for TSGs, homozygously deleted regions within the cancer cell genome are likely to serve as a good landmark (5–9), although biallelic loss is a rare event, and other factors, such as point mutations and epigenetic abnormalities (10), may predominantly contribute to functional inactivation. Therefore, high-resolution mapping of homozygous deletions within the entire genome of cancer cells would be of considerable help in the rapid identification of TSGs. Recently, we have applied an in-house bacterial artificial chromosome (BAC)–based array containing 800 BAC clones (MCG Cancer Array-800; ref. 5) to an array-based comparative genomic hybridization (array-CGH) analysis of OC cell lines, and identified connective tissue growth factor (*CTGF/CCN2*) as a putative ovarian TSG mainly inactivated by DNA methylation from homozygous loss at 6q23 (11). Because (*a*) there is no doubt that carcinoma is the result of the accumulation of multiple somatic genetic and/or epigenetic alterations resulting in either the activation of oncogenes or the inactivation of TSGs and (*b*) homozygous loss is usually small, more TSGs involved in the ovarian carcinogenesis will be identified through the genome-wide search for copy-number changes using arrays with a higher resolution, as shown in our previous studies in various other cancers (12–14).

In the report presented here, we have employed an in-house BAC array with an average spacing of 0.7 Mb (MCG Whole Genome Array-4500), which has ~5.6-fold higher resolution than MCG Cancer Array-800 (5), to a panel of OC cell lines for genomewide copy-number analysis. During the course of these experiments, we identified a novel homozygous loss at 9q33.3 containing angiopoietin-like protein 2 (*ANGPTL2*), the expression of which was absent in some OC cell lines without homozygous loss, although it was present in the normal ovary. To clarify the mechanism and the effect on ovarian carcinogenesis of down-regulated ANGPTL2 expression, we further determined the expression and methylation status of *ANGPTL2* and their clinicopathologic and functional significance in OC.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Cell lines and primary tumors. Twenty-four OC cell lines whose derivation and sources have been previously reported (11) were used. The immortalized normal ovarian epithelial cell line OSE-2a (15), kindly provided by Dr. Hidetaka Katabuchi (Kumamoto University School of Medicine, Kumamoto, Japan), was used as a normal control. All cell lines were maintained in appropriate medium supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. To prepare a conditioned medium, SAS, an oral squamous cell carcinoma cell line that grows in serum-free DMEM/F12 (1:1), was used.

Primary OC tumor samples were obtained during surgery from 100 patients being treated at the National Cancer Center Hospital in Tokyo, with written consent from each patient in the formal style and after approval by the local ethics committee, and were embedded in paraffin for immunohistochemistry. Samples from 45 of these patients were immediately frozen in liquid nitrogen and stored at −80°C until required. DNA of a quality good enough for a methylation analysis was obtained from each of the 45 samples, whereas RNA of a quality good enough for an expression analysis was obtained from only 4 samples. None of the patients had received preoperative radiation or immunotherapy. All patients underwent complete surgical staging, including i.p. cytology, bilateral salpingo-oophorectomy, hysterectomy, omentectomy, and pelvic/para-aortic lymphadenectomy. Aggressive cytoreductive surgery was conducted in patients with advanced disease. Surgical staging was based on the International Federation of Gynecology and Obstetrics staging system: stage I, 53 patients; stage II, 11 patients; stage III, 28 patients; and stage IV, 8 patients.

Array-CGH. Array-CGH using a MCG Whole Genome Array-4500 (5) was carried out as described elsewhere (13). Images acquired by a GenePix 4000B (Axon Instruments) were analyzed with GenePix Pro 6.0 software (Axon Instruments). After normalization, average ratios that deviated significantly (>2 SD) from 0 ($\log_2$ ratio, <−0.4 and >0.4) were considered abnormal.

PCR. Homozygous deletions were detected by genomic PCR (11, 13). For expression analyses, single-stranded cDNA generated from total RNA was amplified with primers specific for each gene (16). The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was amplified at the same time to allow the estimation of the efficiency of cDNA synthesis. For conventional reverse transcription-PCR (RT-PCR), PCR products were electrophoresed, whereas quantitative real-time RT-PCR was done with an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Each assay was conducted in triplicate. All primer sequences are listed in Supplementary Table S1.

Drug treatment. OC cells were cultured with various concentrations of 5-aza 2′-deoxycytidine (5-aza-dCyd) for 5 days and/or 100 ng/mL of trichostatin A (TSA) for the last 12 h.

Methylation analysis. Genomic DNA was treated with sodium bisulfite, and subjected to PCR using primers to amplify regions of interest (Supplementary Table S1). For the combined bisulfite restriction analysis (COBRA), a semiquantitative bisulfite-PCR analysis (17), PCR products were digested with *Bst*UI and electrophoresed. For bisulfite sequencing, PCR products were subcloned and then sequenced. For the methylation-specific PCR (MSP) analysis, sodium bisulfite–treated DNA was subjected to PCR using primer sets specific to the methylated and unmethylated forms of DNA sequences, and PCR products were visualized on 3% agarose gels. DNA from cell lines recognized as unmethylated by bisulfite sequencing was used as negative controls for methylated alleles, whereas DNA from lines recognized as methylated or CpGenome Universal Methylated DNA (Chemicon International) was used as positive controls.

Promoter reporter assay. DNA fragments around the *ANGPTL2* CpG-island were obtained by PCR and ligated into the reporter vector pGL3-Basic (Promega). The reporter assay was performed as described elsewhere (11) using each construct or an empty vector with an internal control pRL-hTK (Promega).

Western blotting. For Western blotting, cell lysates were analyzed as described elsewhere (11). Anti-ANGPTL2, anti–Myc-Tag, and anti-β-actin antibodies were purchased from R&D Systems, Cell Signaling Technology, and Sigma, respectively.

Immunohistochemistry. Indirect immunohistochemistry was performed with formalin-fixed, paraffin-embedded tissue sections as described elsewhere (11). After blocking in 2% normal swine serum, the slides were incubated with an anti-ANGPTL2 antibody (1:500 dilution; R&D Systems) and then reacted with a Histofine simple stain, MAX PO(G) (Nichirei). Antigen-antibody reactions were visualized with 0.2% diaminobenzidine tetrahydrochloride and hydrogen peroxide. The slides were counterstained with Mayer's hematoxylin.

Formalin-fixed HT cells expressing *ANGPTL2* mRNA, >50% of which showed cytoplasmic staining of ANGPTL2 protein, and KF28 cells lacking *ANGPTL2* mRNA expression, none of which showed staining of ANGPTL2 protein, were used as positive and negative controls, respectively. The specificity of the antibody was verified by Western blotting. The percentage of the total cell population that expressed ANGPTL2 was evaluated for each case at ×200 magnification. Expression of ANGPTL2 was graded as either positive (>10% of tumor cell cytoplasm showing immunopositivity, 49 tumors) or negative (<10% of tumor cell cytoplasm showing immunopositivity or no staining, 51 tumors) according to the results in our preliminary analyses (Supplementary Fig. S1).

Growth assay. For colony formation assays (11), a plasmid expressing COOH-terminal Myc-tagged and His-tagged ANGPTL2 (pcDNA3.1-ANGPTL2-Myc-His) was obtained by cloning the PCR product of the full coding sequence of *ANGPTL2* in-frame along with the Myc and 6xHis epitopes into pcDNA3.1 (Invitrogen). pcDNA3.1-ANGPTL2-Myc-His, or the empty vector (pcDNA3.1-mock), was transfected into cells. Cells were stained with crystal violet after 2 weeks of incubation in six-well plates with appropriate concentrations of G418.

To assess the effect of ANGPTL2 on the growth of OC cell lines, cells were treated with the conditioned medium containing ANGPTL2 (18). pcDNA3.1-ANGPTL2-Myc-His or pcDNA3.1-mock was introduced into SAS cells lacking the expression of ANGPTL2. Cells were washed thrice with serum-free medium 24 h after transfection, and then cultured for 4 days. Media were changed everyday. The obtained conditioned media were centrifuged, and supernatants were pooled, concentrated (1:100) with the Amicon Ultra-15 YM-50 (Millipore), sterilized with a Costar Spin-X Centrifuge Tube Filter (Corning), and stored at −80°C prior to use. OC cell lines lacking the expression of *ANGPTL2* were treated with medium containing 0.2% fetal bovine serum and 1% concentrated conditioned medium. The number of viable cells after treatment were assessed by a colorimetric water-soluble tetrazolium salt (WST) assay (11). The cell cycle in ANGPTL2-treated cells was analyzed using fluorescence-activated cell sorting (FACS) as described elsewhere (11).

ANGPTL2-specific small interfering RNA (siRNA; *ANGPTL2*-siRNA) was purchased from Dharmacon. A control siRNA for the luciferase gene (CGUACGCGGAAUACUUCGA, *Luc*-siRNA) was synthesized by Sigma. Each siRNA (50 nmol/L) was introduced into OC cells using LipofectAMINE RNAiMAX (Invitrogen). The number of viable cells 24 to 96 h after transfection was assessed by WST assay.

Statistical analysis. Differences between subgroups were tested with the Mann-Whitney *U* test. Correlations between *ANGPTL2* methylation or expression in primary OCs and the clinicopathologic variables pertaining to the corresponding patients were analyzed for statistical significance with χ^2 or Fisher's exact test. For analysis survival, Kaplan-Meier survival curves were constructed for groups based on univariate predictors, and differences between the groups were tested with the log-rank test. Differences were assessed with a two-sided test and considered significant at the $P < 0.05$ level.

Results

Array-CGH analysis of OC cell lines. In the array-CGH analysis using an MCG Whole Genome Array-4500, frequently detected copy-number gains and losses within the entire genome of 24 OC cell lines (data not shown) were the same as those in our previous

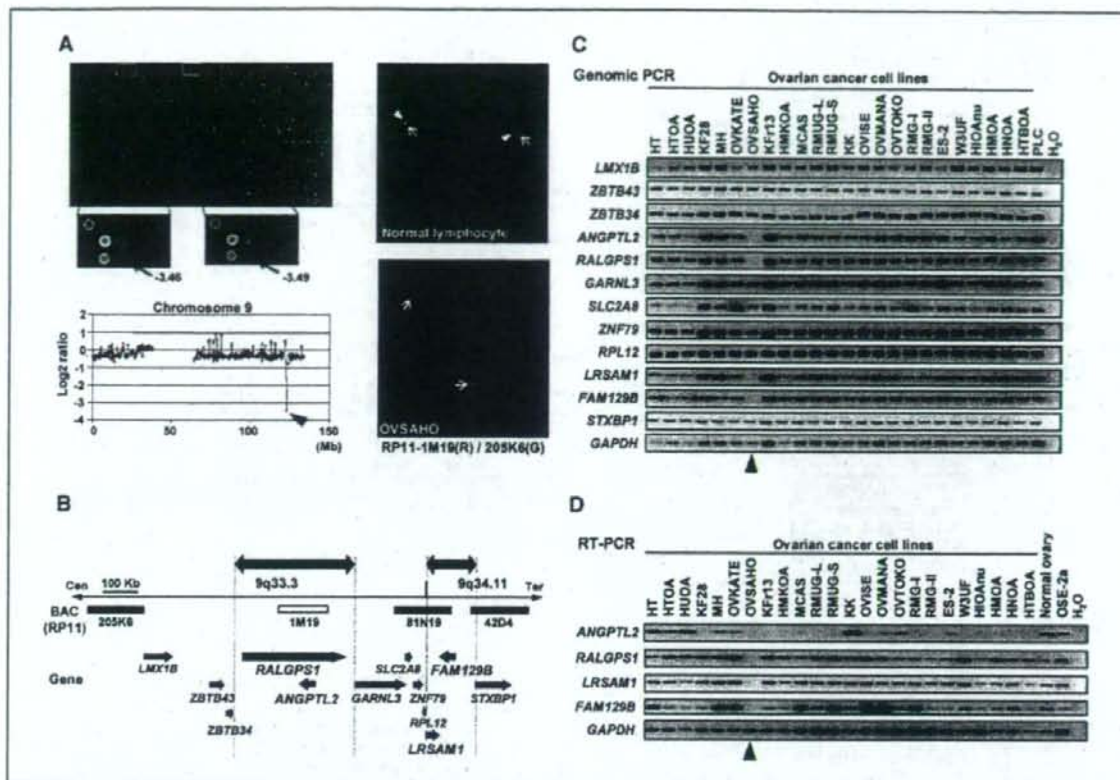

Figure 1. *A*, identification of the 9q33.3 homozygous deletion in the OC cell line. *Top left*, duplicate array-CGH image (MCG Whole Genome Array-4500) of the OVSAHO cell line. A homozygous deletion (copy-number ratio as $\log_2$ ratio) of the BAC clone at 9q33.3 was detected as a clear red signal (*red arrows*). *Bottom left*, representative copy-number profiles of chromosome 9 in OVSAHO cells. *Red arrowhead*, candidate spots (RP11-1M19) showing patterns of homozygous deletion ($\log_2$ ratio <-2). *Right*, FISH image from probe RP11-1M19 (*red signals, arrowheads*) and with RP11-205K6 as a control (*green signals, arrows*) hybridized to metaphase chromosomes from the control normal peripheral lymphocyte (*top*) and OVSAHO cell line (*bottom*). The absence of red signals indicates homozygous loss of sequences within RP11-1M19 in the OVSAHO cell line. *B*, map of 9q33.3-q34.11 covering the region homozygously deleted in the OVSAHO cell line. BAC (RP11-1M19) was homozygously deleted in the array-CGH analysis (*vertical white bar*). The homozygously deleted region in OVSAHO cells, as determined by genomic PCR analysis (*vertical red closed arrow*). Ten genes located within this region (*red arrows*, homozygously deleted genes; *black arrows*, retained genes) showing the positions and directions of transcription. *C*, genomic PCR analyses of genes located around the 9q33.3-9q34.11 homozygously deleted region in OC cell lines. Homozygous deletions of *ANGPTL2, RALGPS1, LRSAM1*, and *FAM129B* but not *LMX1B, ZBTB43, ZBTB34, GARNL3, SLC2A8, ZNF79, RPL12*, and *STXBP1* were detected in one OC cell line (*OVSAHO, arrowhead*) by genomic PCR. *D*, mRNA expression of *ANGPTL2, RALGPS1, LRSAM1*, and *FAM129B* in OC cell lines and the normal ovary and normal ovarian epithelial cell–derived cell line OSE-2a, detected by RT-PCR. *Arrowhead*, a cell line with the homozygous deletion indicated in the genomic PCR analysis. Expression of *RALGPS1, LRSAM1*, and *FAM129B* mRNAs was observed to some degree in most OC cell lines, whereas *ANGPTL2* showed frequent silencing. Notably, 18 of the 23 cell lines (78%) without a homozygous deletion of *ANGPTL2* showed decreased expression.

study (11). Compared with the MCG Cancer Array-800, we identified more homozygous deletions ($\log_2$ ratio <-2) and high-level amplifications ($\log_2$ ratio > 2), which are likely to be landmarks of TSGs and oncogenes, respectively, using the MCG Whole Genome Array-4500: homozygous deletions at 4q, 6q, 8q, 9p, and 9q (Supplementary Table S2), and amplifications at 2q, 11q, and 19q (Supplementary Table S3). All these alterations were confirmed by fluorescence *in situ* hybridization (Fig. 1*A*; data not shown). Among them, the homozygous loss at 9q33.3 observed in OVSAHO cells had never been previously documented in OC, prompting us to examine whether genes located within this region are involved in the pathogenesis of OC.

Identification of target genes involved in homozygous deletion at 9q33.3. To define the extent of the homozygous loss at 9q33.3 in OVSAHO cells and to identify other OC lines harboring a cryptic homozygous loss in this region, we performed genomic PCR with 12 genes (*LMX1B, ZBTB43, ZBTB34, RALGPS1, ANGPTL2, GARNL3, SLC2A8, ZNF79, RPL12, LRSAM1, FAM129B*, and *STXBP1*; Fig. 1*B*) located around RP11-1M19 according to information archived by genome databases.[9,10] We detected a complete loss of *ANGPTL2, RALGPS1, LRSAM1*, and *FAM129B* only in OVSAHO cells, whereas *LMX1B, ZBTB43, ZBTB34, GARNL3, SLC2A8, ZNF79, RPL12*, and *STXBP1* were retained in this cell line (Fig. 1*C*), indicating that the homozygous deletion has a structurally complicated pattern, and its total size is ~0.55 Mb at maximum.

[9] http://www.ncbi.nlm.nih.gov/
[10] http://genome.ucsc.edu/

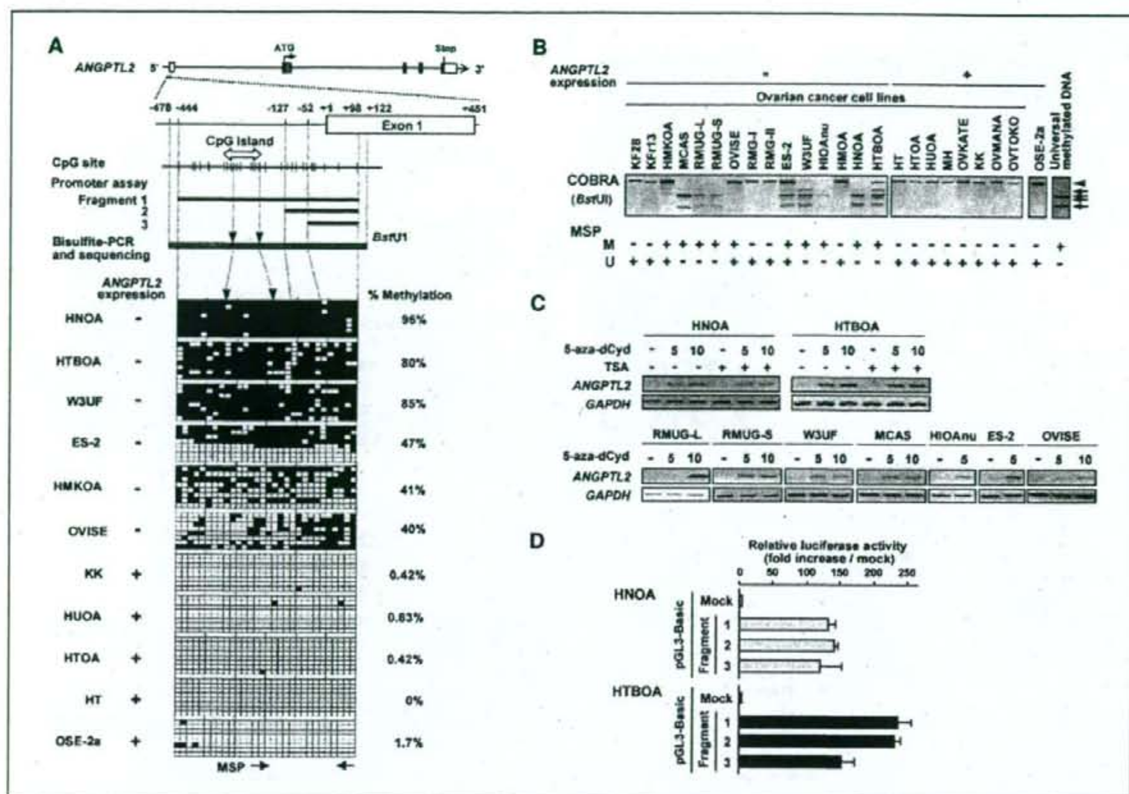

Figure 2. Methylation status of the *ANGPTL2* CpG-rich region in OC cell lines. *A*, schematic map of the CpG-rich region containing the CpG-island (*closed white arrow*) around exon 1 of *ANGPTL2* and representative results of bisulfite sequencing. CpG sites (*vertical ticks*), exons (*open box*), and the transcription-start site (*marked at +1*) on the expanded axis. *Thick black lines*, the fragments examined in a promoter assay; *horizontal gray bar*, the regions examined in the COBRA and bisulfite sequencing; *black downward arrowheads*, restriction sites for *Bst*UI in COBRA. Representative results of bisulfite sequencing of the *ANGPTL2* CpG-rich region examined in *ANGPTL2*-expressing OC cell lines (+) and nonexpressing OC cell lines (−). Each square indicates a CpG site: *open squares*, unmethylated; *solid squares*, methylated. *Arrows*, PCR primers for MSP. *B*, representative results of the COBRA of the *ANGPTL2* CpG-island in OC cell lines after restriction with *Bst*UI. *Arrows*, fragments specifically restricted at sites recognized as methylated CpGs; *arrowheads*, undigested fragments indicating unmethylated CpGs. Results of the MSP analysis are also shown. *M*, methylated allele; *U*, unmethylated allele. Representative images of MSP are shown in Supplementary Fig. S2. CpGenome Universal Methylated DNA (Chemicon International) was used as positive controls for methylation analyses. *C*, representative results of RT-PCR to reveal restored *ANGPTL2* expression after demethylation in cell lines lacking its expression. *Top*, restored *ANGPTL2* expression in HNOA and HTBOA cell lines after treatment with 5-aza-dCyd (5 or 10 μmol/L) for 5 d with or without TSA (100 ng/mL) for the last 12 h. Notably, almost no effect of TSA treatment on *ANGPTL2* expression was observed in cells either with or without 5-aza-dCyd treatment. *Bottom*, restored *ANGPTL2* expression in RMUG-L, RMUG-S, W3UF, MCAS, HIOAnu, ES-2, and OVISE cell lines after treatment with 5-aza-dCyd, which showed reduced expression of *ANGPTL2* mRNA (Fig. 1*D*) and a methylated pattern (Fig. 1*B*). *D*, promoter activity of the *ANGPTL2* CpG-rich region around the CpG-island. pGL3 basic empty vectors (mock) and constructs containing one of three different sequences around the highly methylated region of *ANGPTL2* (fragments 1–3; 542, 225, and 150 bp in size, respectively, in *A*) were transfected into HNOA and HTBOA cells. Luciferase activity was normalized vs. an internal control. *Columns*, means for three separate experiments, each performed in triplicate; *bars*, SD.

Loss of *ANGPTL2* expression in OC cell lines. Next, we determined the mRNA expression levels of *ANGPTL2, RALGPS1, LRSAM1*, and *FAM129B* by RT-PCR in all 24 OC lines, normal ovary, and the OSE-2a cell line. *RALGPS1, LRSAM1*, and *FAM129B* were expressed in most of the OC lines at levels similar to or higher than those in normal ovary and/or the OSE-2a cell line (Fig. 1*D*). On the other hand, *ANGPTL2* mRNA was frequently silenced in OC lines without the homozygous deletion (18 of 23, 78%; Fig. 1*D*), but was expressed in normal ovary and OSE-2a cells, suggesting that this gene is likely to be the most probable target for inactivation through mechanisms other than genomic deletion in OC cells. Because aberrant methylation within the CpG-island around the transcription start site (TSS) of genes is known to be one of the key mechanisms by which TSGs can be silenced (9), and the CpGPLOT program[11] identified the CpG-island around the TSS of *ANGPTL2*, we focused on *ANGPTL2* for further DNA methylation analyses. None of the two lines that had shown a hemizygous loss around *ANGPTL2* in array-CGH exhibited a decreased expression of this gene (data not shown).

Methylation of the *ANGPTL2* CpG-island in OC cell lines. To show the potential role of methylation within the CpG-island in the silencing of *ANGPTL2*, we first assessed the methylation status of each CpG site around the *ANGPTL2* CpG-island (Fig. 2*A*) in OC lines with or without *ANGPTL2* expression and the OSE-2a cells, by

[11] http://www.ebi.ac.uk/emboss/cpgplot/

means of bisulfite sequencing. CpG sites around the *ANGPTL2* CpG-island tended to be extensively (HNOA, HTBOA, and W3UF) or partially (ES-2, OVISE, and HMKOA) methylated in the non-expressing cell lines, whereas *ANGPTL2*-expressing OC lines (KK, HUOA, HTOA, and HT) and OSE-2a cells were almost unmethylated (Fig. 2*B*). We then compared the methylation and expression status of *ANGPTL2* in a larger number of OC lines by COBRA covering the region around the CpG-island (Fig. 2*A*) and MSP designed to target the region around TSS (Fig. 2*A*), because two *Bst*UI restriction sites for COBRA may fail to detect DNA methylation around TSS. Consistent with the results of bisulfite sequencing, no methylated allele was detected among any of the OC cell lines with *ANGPTL2* expression and OSE-2a cells with either method (Fig. 2*B*; Supplementary Fig. S2). On the other hand, a methylated allele was detected in 10 of 15 OC cell lines lacking *ANGPTL2* expression by either method, although some of these cell lines retained an unmethylated allele. Five of the 15 OC cell lines (KF28, KFr13, RMG-I, RMG-II, and HMOA) lacking *ANGPTL2* expression were found to have only an unmethylated allele by either method, suggesting that mechanisms other than DNA methylation, including epigenetic silencing of transcription factors regulating *ANGPTL2* transcription, or upstream components of the signaling pathway activating *ANGPTL2* expression, also contribute to the silencing of *ANGPTL2* directly or indirectly.

To investigate whether DNA demethylation could restore the expression of *ANGPTL2* mRNA, we treated OC cells lacking *ANGPTL2* expression with 5-aza-dCyd. The induction of *ANGPTL2* mRNA expression occurred after treatment with 5 or 10 μmol/L of

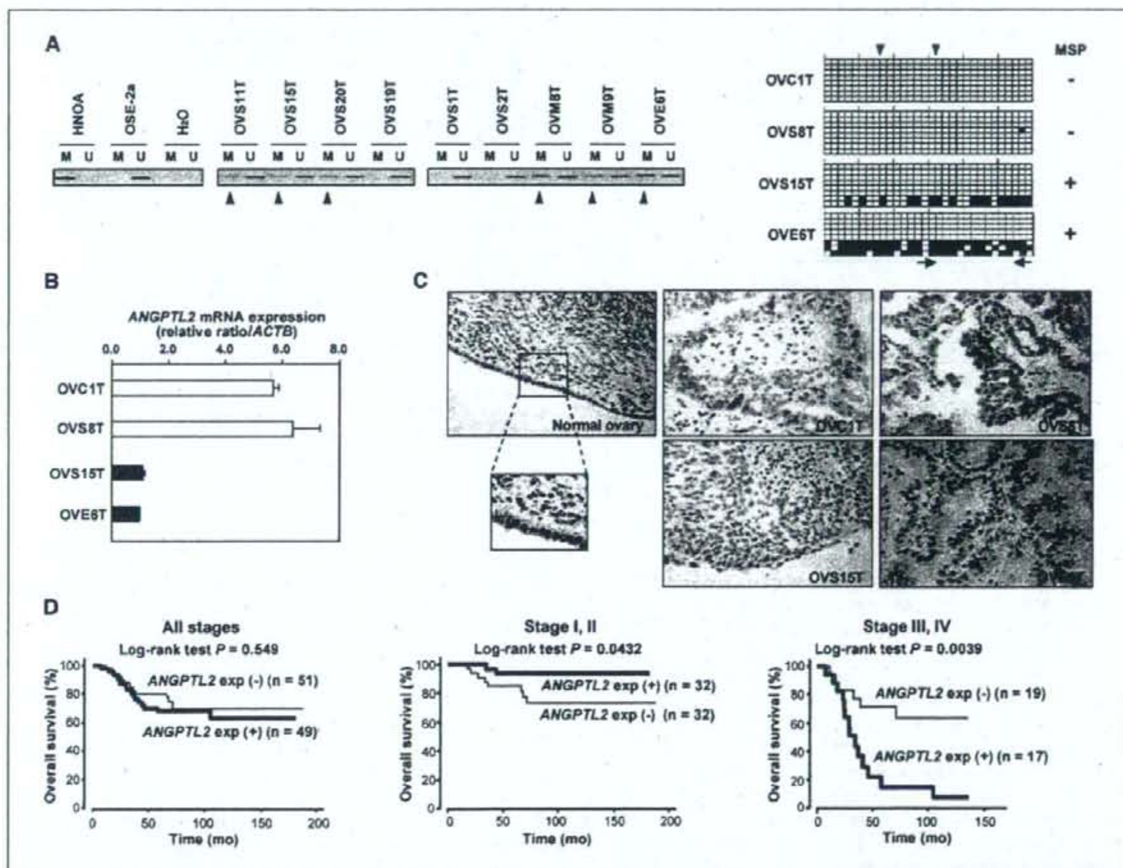

Figure 3. Methylation and expression status of *ANGPTL2* in primary tumors of OC. *A*, representative results of a MSP analysis (*left*) and bisulfite sequencing (*right*) of the *ANGPTL2* promoter region in primary OC tissues. *Left*, in the MSP analysis, parallel amplification reactions were performed using primers specific for unmethylated (*U*) or methylated (*M*) DNA. *Right*, methylation status of *ANGPTL2* determined by bisulfite sequencing in tumor samples. See legend of Fig. 2*A* for interpretation. *B*, representative results indicating an inverse correlation between methylation status of *ANGPTL2* determined by MSP and mRNA expression status determined by quantitative real-time RT-PCR in four primary tumors. *C*, representative results of immunohistochemical staining of ANGPTL2 protein in normal human ovarian epithelial cells and primary OC tumors. ANGPTL2 expression is shown in normal ovarian epithelial cells (*top left*). In primary OC, strong (*OVC1T* and *OVS8T*) or very weak (*OVS15T* and *OVE8T*) expression of ANGPTL2 was observed (original magnification, ×200). *D*, Kaplan-Meier curves for overall survival rates of patients at all stages (*left*), stage I and II (*middle*), and stage III and IV OC (*right*). In overall survival, no significant difference was observed between the patients with positive ANGPTL2 expression and those with negative ANGPTL2 expression in all stages ($P = 0.600$). In stage I and II disease, however, negative ANGPTL2 immunoreactivity in tumor cells was significantly associated with a worse overall survival ($P = 0.0432$), whereas positive ANGPTL2 immunoreactivity was significantly associated with a worse overall survival in stage III and IV disease ($P = 0.0039$).

Table 1. Correlation between clinical background and expression of ANGPTL2 protein

	Total (n)	Expression of ANGPTL2, n (%)*	P †
Total	100	49 (49)	
Age (y)			
<60	69	34 (49)	0.935
≥60	31	15 (48)	
FIGO stage			
I, II	64	32 (50)	0.790
III, IV	36	17 (47)	
Histologic type			
Serous	41	16 (39)	0.168
Mucinous	14	11 (79)	
Clear cell	34	17 (50)	
Endometrioid	11	5 (45)	
Optimal surgery ‡			
Optimal (<2 cm)	82	38 (46)	0.217
Suboptimal (≥2 cm)	13	9 (69)	
Peritoneal cytology ‡			
Positive	46	23 (50)	0.765
Negative	49	23 (47)	
Methylation§			
Positive	11	2 (18)	**0.0402**
Negative	34	19 (56)	

NOTE: Statistically significant values are in boldface type.
Abbreviation: FIGO, International Federation of Gynecology and Obstetrics.
*ANGPTL2 protein expression was evaluated by immunohistochemical analysis as described in Materials and Methods.
† P values are from χ^2 or Fisher's exact test and were statistically significant when <0.05 (two-sided).
‡ No information was available in five cases.
§ No high-quality DNA for methylation analysis was available in 55 cases.

5-aza-dCyd in HNOA and HTBOA cell lines (Fig. 2*C*). In addition, treatment with TSA had no effect on the *ANGPTL2* mRNA expression with or without 5-aza-dCyd in those cell lines, suggesting that DNA methylation is of primary importance for epigenetic silencing in OC cell lines. Restoration of *ANGPTL2* expression by 5-aza-dCyd was also observed in other cell lines, such as RMUG-L, RMUG-S, W3UF, MCAS, HIOAnu, ES-2, and OVISE, lacking expression and showing methylation of *ANGPTL2* in the COBRA and/or MSP analyses (Fig. 2*C*). Among *ANGPTL2* nonexpressing cell lines showing no methylation pattern in both COBRA and MSP analyses, almost no restoration of *ANGPTL2* expression was observed in RMG-I, RMG-II, and KFr13 cell lines, whereas a little restoration was observed in HMOA and KF28 cell lines by 5-aza-dCyd alone (Supplementary Fig. S3). TSA alone did not affect the expression of *ANGPTL2* in those lines, but induced expression in combination with 5-aza-dCyd in some cell lines (Supplementary Fig. S3).

Promoter activity of the sequence around the *ANGPTL2* CpG-island. Because the sequence around the *ANGPTL2* CpG-island seems to be a target for methylation-mediated gene silencing, we tested three fragments designed according to the results of bisulfite sequencing for promoter activity (fragments 1–3 in Fig. 2*A*). All three fragments showed a remarkable increase in transcriptional activity in HNOA and HTBOA lines (Fig. 2*D*), suggesting that the region around the *ANGPTL2* CpG-island, especially the sequence around TSS, contains critical sequences for basal gene expression and may be a target for methylation.

Analysis of *ANGPTL2* methylation and expression in primary OC tumors. To determine if the aberrant methylation of *ANGPTL2* also takes place in primary tumors of OC, we did MSP analysis with primer sets targeting the sequence around the most frequently methylated sites (Fig. 2*A*) in 45 primary cases. Consistent with the results of bisulfite sequencing and COBRA (Fig. 2*A* and *B*), a representative cell line lacking *ANGPTL2* expression (HNOA) was methylated, whereas the *ANGPTL2*-expressing cell line (OSE-2a) was unmethylated (Fig. 3*A*). We detected *ANGPTL2* hypermethylation in 11 of the 45 primary OCs (24%; Supplementary Table S4; Fig. 3*A*; data not shown). To quantitatively confirm the results of MSP analysis, we performed bisulfite sequencing in some representative cases. Aberrant methylation in a pattern similar to that observed in OC lines lacking *ANGPTL2* expression was observed in OC tissues, which showed a methylation pattern in the MSP, whereas tumors with an unmethylated pattern in the MSP showed hypomethylation in bisulfite sequencing (Fig. 3*A*; data not shown).

In four cases of OC in which tumor samples were available for triple analyses, i.e., a methylation analysis, real-time RT-PCR, and immunohistochemistry, we compared the expression status of *ANGPTL2* with its methylation status (Fig. 3*A–C*). Methylation-positive tumors tended to express fewer *ANGPTL2* mRNA than methylation-negative tumors even in this small number of cases. In addition, methylation-negative tumors showed positive ANGPTL2 immunostaining in >10% of cancer cells, whereas methylation-positive tumors showed positive ANGPTL2 staining in <10% of cancer cells, suggesting that methylation is one of the mechanisms suppressing the mRNA and protein expression of ANGPTL2. In neighboring nonneoplastic epithelia, staining for ANGPTL2 was observed (Fig. 3*C*).

Association between expression of ANGPTL2 protein and clinicopathologic characteristics in primary cases. To clarify the clinical significance of ANGPTL2 in OC, the expression level of ANGPTL2 protein in 100 primary OC tissues was evaluated by immunohistochemistry. Negative and positive immunoreactivities of ANGPTL2 (Supplementary Fig. S1) were found in 51 (51%) and 49 (49%) of 100 cases, respectively. The relationship between the expression of ANGPTL2 protein and the clinicopathologic characteristics is summarized in Table 1. In 45 cases from which high-quality DNA was available for a MSP, the methylation status of *ANGPTL2* was inversely correlated with the expression of ANGPTL2 protein (P = 0.0402).

ANGPTL2 protein expression in each sample was not associated with age, histologic subtype, tumor staging, the age of patients, the results of surgery, or peritoneal cytology, although data were not fully available for some of those variables. Methylation status in each sample was not associated with these characteristics either (Supplementary Table S4). In overall survival (Fig. 3*D*), no significant difference was observed between the patients with negative and positive ANGPTL2 in all stages. In stage I and II disease, however, negative ANGPTL2 immunoreactivity in tumor cells was significantly associated with a worse overall survival (P = 0.0432), whereas positive ANGPTL2 immunoreactivity was significantly associated with a worse overall survival in stage III and IV disease (P = 0.0039).

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Cap43/NDRG1/Drg-1 is a molecular target for angiogenesis and a prognostic indicator in cervical adenocarcinoma

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Abstract

Cap43 is a nickel- and calcium-inducible gene that plays important roles in the primary growth of malignant tumors, as well as in invasion and metastasis, most likely through its ability to induce cellular differentiation. This study investigated associations of Cap43 expression with angiogenesis and other clinicopathological factors in cervical adenocarcinoma. The clinical records of 100 women who underwent surgery for cervical adenocarcinoma were reviewed retrospectively. Microvessel density and the expression of Cap43 and VEGF in the surgical specimens were evaluated immunohistochemically. The Cap43 expression level was significantly associated with angiogenesis, tumor diameter, stromal invasion, lymphovascular space invasion, lymph node metastasis, and histopathological differentiation. Kaplan–Meier analysis showed a significant association between the Cap43 expression level and survival: high Cap43 expression was related to poor survival. Our results suggest that increased expression of Cap43 is associated with angiogenesis and may be a poor prognostic indicator in women with cervical adenocarcinoma.
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Keywords: Cap43; Cervical adenocarcinoma; Angiogenesis; Prognosis

1. Introduction

The Cap43 gene is a nickel- and calcium-inducible gene [1], identical to the previously described N-myc downstream-regulated gene 1 (NDRG1). It

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is one of the four closely related genes (NDRG1-4) whose expression is down-regulated by c-myc or the N-myc/Max complex [2–5]. Cap43 is also identical to the homocysteine-inducible gene, whose expression is reduced in tumor cells (RTP/rit42) [6], and to the differentiation-related gene-1 (Drg-1) [7]. The protein encoded by Cap43 has a molecular weight of 43 kDa. It has three unique 10-amino acid tandem-repeat sequences at its carboxyl terminal and is phosphorylated by protein kinase A [8].

The functions of Cap43 remain poorly understood. Expression of the Cap43 gene has been strongly associated with nickel, cobalt, oxidative stress, hypoxia, phorbol esters, vitamins A and D, steroids, histone deacetylase-targeting drugs, homocysteine, β-mercaptoethanol, tunicamycin, and lysophosphatidylcholine, as well as with oncogenes (N-myc and c-myc) and the products of tumor-suppressor genes (p53 and VHL) [1,2,6,9,10]. Cap43 is expressed in most organs, most prominently in the prostate, ovary, colon, and kidney. Its expression in the kidney, brain, liver, and gut is actively modulated during postnatal development [2,3,11,12], suggesting that Cap43 plays a key role in organ maturation.

Transfection studies by Kurdistani and colleagues demonstrated that Cap43 inhibits the primary growth of human breast, prostate, and bladder cancer cell lines and suppresses the anchorage-independent growth of these cell lines in soft agar [6]. Moreover, the overexpression of Cap43 markedly promotes the growth of human pancreatic cancer xenografts in mice, but not of pancreatic cancer cells in culture [13]. The survival rate of patients with pancreatic cancer whose tumors expressed high levels of Cap43 was found to be significantly higher than that of patients whose tumors expressed low levels of Cap43 [13]. In another study, low tumor Cap43 expression was strongly associated with poor outcomes in women with breast cancer [14]. Chua and colleagues recently reported that Cap43 overexpression significantly correlates with tumor differentiation, vascular invasion, and overall survival in patients with hepatocellular carcinoma, suggesting that increased Cap43 expression may be a useful indicator of tumor aggressiveness and prognosis [15]. Taken together, the above findings suggest that Cap43 may have tissue-of-origin-specific functions in human malignancies [16].

The incidence of invasive cervical cancer has decreased in developed countries, presumably because of intensive national screening programs. This declining incidence is attributed primarily to a decrease in squamous cell carcinoma, whereas the incidence of adenocarcinoma has remained stable or risen slightly [17,18]. The prevalence of adenocarcinoma among women with cervical cancer has increased from 5% to 13% in the 1950s to 20% in the 1990s [19,20]. Recent studies attribute this rise to an increased incidence of cervical adenocarcinoma among young women [21,22]. Cervical adenocarcinoma is associated with unfavorable outcomes, attributed to late detection on Papanicolaou smears, a poorer response to radiotherapy than squamous cell carcinoma, or the inclusion of subtypes with particularly poor outcomes, such as clear cell carcinoma [23]. New tumor markers that can be used to predict outcomes predictors have been identified by numerous studies, including immunohistochemical analyses [24–26].

Angiogenesis is an important pathological aspect of tumor growth and chronic inflammatory diseases [27]. Of the various angiogenesis factors identified to date, vascular endothelial growth factor (VEGF)-A plays a key role in pathological angiogenesis, including that required for the rapid growth of solid tumors. Antiangiogenesis agents targeting VEGF-A and VEGF-receptor 2 have been developed and are currently used clinically [28,29]. In a previous study, we demonstrated that higher tumor Cap43 expression is associated with higher tumor microvessel density (MVD) than lower tumor Cap43 expression in patients with pancreatic cancer [13]. Angiogenesis in cervical carcinoma has been shown to be inversely related to survival [30,31]. Kaku and colleagues [32] demonstrated a significant correlation of MVD with both progression-free survival and overall survival in cervical adenocarcinoma.

In this study we immunohistochemically evaluated the intensity of Cap43 expression in patients with stage I or II cervical adenocarcinoma according to the staging system of the International Federation of Gynecology and Obstetrics (FIGO). We also examined correlations of Cap43 staining intensity with angiogenesis and other clinicopathological factors.

2. Materials and methods

2.1. Patients and treatment

Between 1990 and 2005, a total of 100 patients with stage I or II cervical adenocarcinoma underwent surgery at Kurume University Hospital and National Cancer

Center Hospital. The procedure was radical hysterectomy in 93 patients and simple abdominal hysterectomy in the other seven. Pelvic lymphadenectomy and para-aortic lymph node biopsy were performed in all patients. Patients with deep stromal invasion, lymph node metastasis, or both were considered candidates for postoperative adjuvant therapy. After surgery, 23 patients received postoperative adjuvant radiotherapy, and 5 received adjuvant platinum-containing chemotherapy. For external beam radiotherapy, a dose of 50.4 Gy was delivered to the entire pelvis. Intracavitary brachytherapy was performed if the surgical margin in the vaginal cuff was histologically positive or if the free margin was <1 cm.

2.2. Immunohistochemical staining

All specimens were fixed in 10% formalin and embedded in paraffin wax. Tissue sections 4 μm thick were mounted on slides, deparaffinized, rehydrated, and heated in a microwave oven for 60 min in CCI buffer. Immunohistochemical staining was performed using a Ventana NX automated immunohistochemistry system (Ventana Medical Systems, Tucson, AZ, USA) and polyclonal primary antibodies to Cap43 (produced in our laboratory), [10,33] VEGF (upstate, Cosmo Bio Co. Ltd., Lake Placid, NY, USA; dilution 1:50), and CD34 (Nichirei, Tokyo, Japan; dilution 1:1). A preliminary study of Cap43 immunohistochemical staining of cervical adenocarcinoma revealed that only the membrane of tumor cells stained positively; normal glands and the nuclei and cytoplasm of tumor cells stained negatively. Since the staining intensity varied, we considered the staining intensity of the tumor cell membranes to represent the expression intensity of Cap43 in the cervical adenocarcinoma specimens. The intensity of membrane staining was scored as follows: no staining, 0; dotted pattern staining, 1+; weak or moderate circumferential staining in >10% of the tumor cells, 2+; strong circumferential staining in >10% of the tumor cells, 3+ (see Fig. 1A–D). To statistically analyze the patients' survival curves (data not shown), we classified Cap43 expression scores of 0, 1+, and 2+ as low Cap43 expression, and scores of 3+ as high Cap43 expression. VEGF expression in the tumor cells was evaluated according to the following semiquantitative scoring system: no staining at all or staining in <10% of the tumor cells, 0; light staining in >10% of the tumor cells, 1+; moderate staining in >10% of the tumor cells, +2; and dark staining in >10% of the tumor cells, +3. Staining of the tumor stroma was ignored in this assessment (see Fig. 1E–H). All procedures were performed by one gynecological oncologist and two pathologists who were blinded to clinical outcomes in this series of patients. Discordant results among the investigators were re-evaluated. MVD was calculated on the basis of the immunohistochemical expression of CD34. For each sample, the mean number of microvessels was calculated for five vascular hotspots to assess the MVD for each case. Only CD34 staining in tumor areas was reviewed, and endothelial cell clusters of two or more cells were considered a single, countable microvessel (see Fig. 1I and J). All counts were made by three independent observers who had no knowledge of the corresponding clinicopathological data.

2.3. Statistical analysis

Statistical calculations were performed with the SAS version 9.1.3 (SAS Institute, Cary, NC, USA) software package. The Kaplan–Meier method was used to calculate the progression-free survival rate and overall survival rate; prognostic significance was evaluated by the log-rank test. The Mann–Whitney *U*-test was used to compare continuous variables. *P* values for correlations of Cap43 expression with VEGF expression and other clinicopathological factors were calculated with Fisher's exact test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Patient characteristics

The patients' characteristics are shown in Table 1. The median follow-up time was 51.3 months. At the time of the analysis, tumor recurrence had been diagnosed in 30 patients, and 25 patients had died. Table 2 shows the Cap43 expression, VEGF expression, and microvessel density.

3.2. Correlation between Cap43 expression and angiogenesis

High Cap43 expression correlated with high VEGF expression (Table 3). Immunohistochemical staining analysis showed that median MVD was 39.4 in the specimens with high Cap43 expression and 26.1 in the specimens with low Cap43 expression. MVD correlated with the intensity of Cap43 expression ($P < 0.0001$, Fig. 2). These results suggested that high Cap43 expression was closely associated with high angiogenic activity in cervical adenocarcinoma.

3.3. Correlation between Cap43 expression and clinicopathological factors

High Cap43 expression significantly correlated with tumor diameter, stromal invasion, lymphovascular space invasion, lymph node metastasis, and histopathological differentiation, but not with FIGO stage (Table 4).

3.4. Correlation between Cap43 expression and survival time

The median progression-free survival time was 52.4 months in patients with tumors showing low Cap43

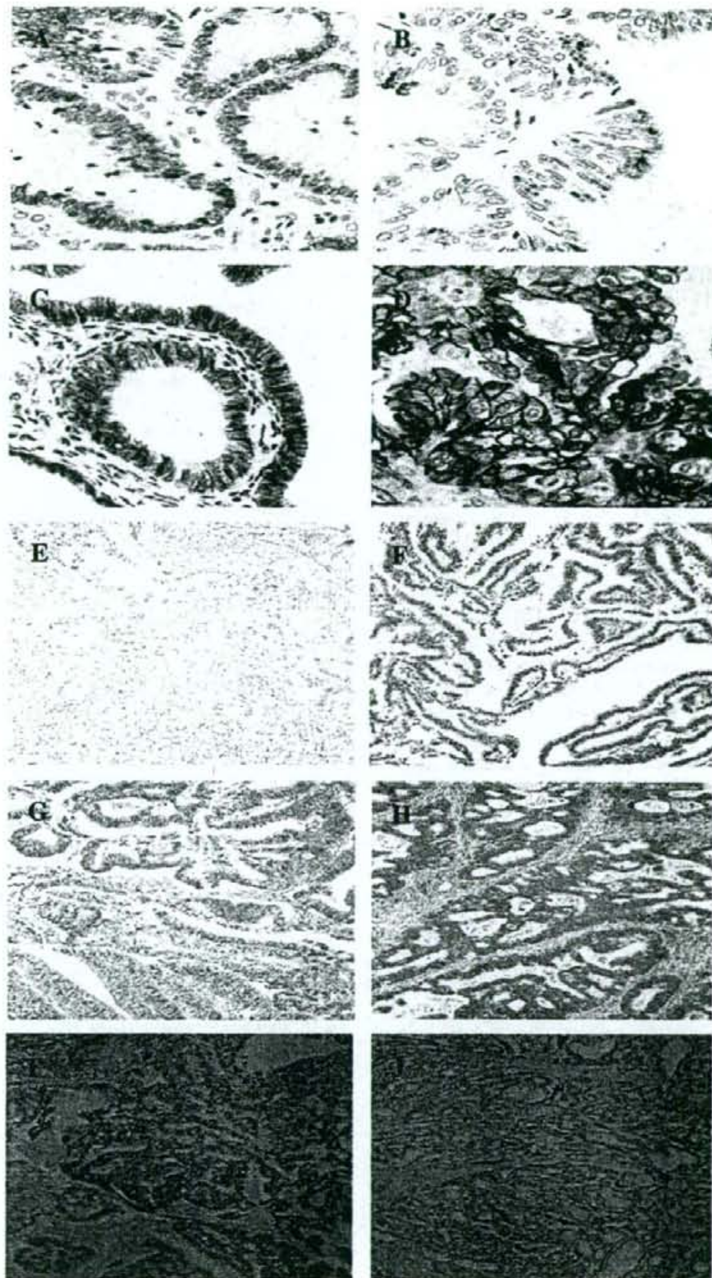

Fig. 1. Representative examples of Cap43 staining in cervical adenocarcinoma. (A) No staining; (B) dotted pattern staining (1+); (C) weak or moderate circumferential staining (2+); (D) strong circumferential staining (3+) (original magnification A–D ×400). Immunohistochemical staining for VEGF expression: (E) no immunostaining (0); (F) light staining (1+); (G) moderate staining (+2); (H) dark staining (+3) (original magnification E–H 200×). Immunohistochemical staining for anti-CD34 antibody. Tumor areas with low vessel density (I) and high vessel density (J) are shown (original magnification I, J 100×).

Table 1
Patients Characteristics ($n = 100$)

Age (years) median (range)	49 (29–74)
<35	10
35–50	45
⩾50	45
FIGO stage	
I	82
II	18
Tumor diameter (mm) median (range)	30 (4–118)
Depth of stromal invasion (mm) median (range)	11 (1–21)
Differentiation	
Well	80
Moderate	11
Poorly	9
Histopathology	
Endocervical type	60
Endometrioid type	33
Intestinal type	2
Serous	3
Clear cell	2

Table 2
Cap43 expression, VEGF expression and MVD

	No. of patients
Immunohistochemical expression of Cap43 score	
0	13
1+	20
2+	35
3+	32
Immunohistochemical expression of VEGF score	
0	13
1+	23
2+	40
3+	24
Microvessel density median (range)	30.2 (8.4–68.1)

Table 3
Correlation between Cap43 expression and VEGF expression

Factor	Cap43 expression		
	Low	High	*P* value
VEGF expression			
Low	56	20	0.0439
High	12	12	

expression, as compared with 27.3 months in those with tumors showing high Cap43 expression ($P = 0.0017$). The median overall survival time was 54.1 months in patients with tumors showing low Cap43 expression, as compared with 36.4 months in those with tumors showing high Cap43 expression ($P = 0.0018$). Kaplan–Meier analysis showed that the intensity of Cap43 expression was significantly associated with survival; high Cap43 expression was associated with unfavorable outcomes (Fig. 3).

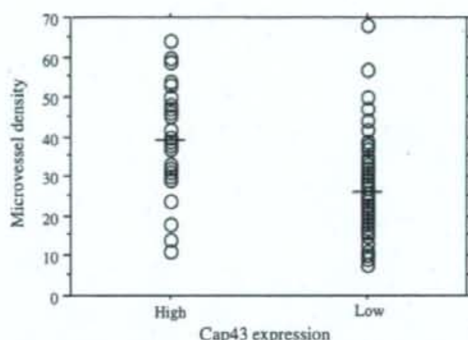

Fig. 2. Correlation between Cap43 expression and microvessel density (MVD). Median MVD was 39.4 in tumors with high Cap43 expression and 26.1 in tumors with low Cap43 expression ($P < 0.0001$).

Table 4
Clinicopathological significance of Cap43 expression

Factor	Cap43 expression		*P* value
	Low	High	
Stage			
I	57	25	0.5788
II	11	7	
Tumor diameter			
<40 mm	51	14	0.0034
⩾40	17	18	
Stromal invasion			
<2/3	42	8	0.0011
⩾2/3	26	24	
Lympho vascular space invasion			
Negative	40	9	0.0053
Positive	28	23	
Lymph node metastasis			
Negative	55	16	0.0022
Positive	13	16	
Differentiation			
Well	60	20	0.0060
Moderate, poorly	8	12	

4. Discussion

Our study showed that the intensity of Cap43 expression was significantly associated with tumor angiogenesis and other poor prognostic factors in cervical adenocarcinoma. Survival analysis showed that high tumor expression of Cap43 was associated with poor progression-free survival and overall survival.

The controversy surrounding the relevance of Cap43 expression in cancer may be attributed in

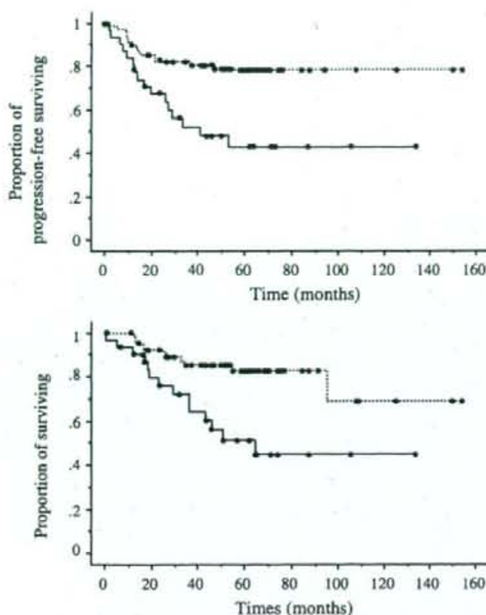

Fig. 3. Kaplan–Meier analysis of progression-free survival (above) and overall survival (bottom) according to Cap43 expression levels in cervical adenocarcinoma. High Cap43 expression (solid line) was associated with significantly poorer outcomes ($P = 0.0017$ and $P = 0.0018$, respectively) than was low Cap43 expression (dotted line). Bold dots indicate censored cases.

part to the fact that Cap43 expression is highly influenced by pleiotropic factors and stimuli, including various metal ions, hypoxia, oncogenes, tumor-suppressor genes, hormones, and vitamins. The expression of Cap43 in patients with cancer thus depends on which factor predominates in a particular case [16]. For example, Cap43 expression in prostate cancer cells is influenced by androgens [34], whereas that in breast cancer cells depends primarily on estradiols [14]. Cap43 expression may vary greatly according to the presence or absence of hormone dependence in hormone-susceptible cancers such as prostate cancer and breast cancer. However, confirmation of possible roles of Cap43 in human malignancies must await the results of future studies that comprehensively evaluate various biologic factors intrinsically related to different types of cancer.

We immunohistochemically studied the intensity of Cap43 expression in surgical specimens. Three patterns of Cap43 expression were observed, consistent with the results of Caruso and colleagues [35]: intense, predominantly membranous staining; intense nucleocytoplasmic localization; and low or undetectable expression. These different patterns of Cap43 expression might be attributed to differences among tumors in factors that either stimulate or inhibit its expression.

Recent advances in cancer research have revealed the importance of angiogenesis to cancer progression. Among the various angiogenic factors identified to date, VEGF and MVD are know to have a pivotal role in tumor angiogenesis and to participate in neovascularization by promoting the differentiation of vascular endothelial cells and increasing capillary permeability. Correlations between neovascularization and metastasis or poor outcomes have been reported in various cancers [36–39]. Previous retrospective studies have reported finding that VEGF and MVD are independent prognostic factors in cervical adenocarcinoma [32]. Our results showed that the significant association of VEGF and MVD with the intensity of Cap43 expression was closely related to high angiogenic activity in cervical adenocarcinoma.

We evaluated Cap43 expression on the basis of the intensity of cellular membrane staining. Cap43 is most often localized in the nucleus, cytoplasm, cell membrane, and intracellular organelles [11]. During the differentiation of various organs, localization of Cap43 may vary between the nuclear membrane and cytoplasm [12,40]. However, Cap43 proteins appear to have no transmembrane domain, signal sequence, or endoplasmic reticulum retention sequence [9]. Cap43 has more than seven phosphorylation sites, two of which are susceptible to protein kinase A and calmodulin kinase II [8]. Recent studies have clearly demonstrated which sites bind to each kinase [16]. These findings suggest that Cap43 has a regulatory role in cells. This regulatory role as well as the cellular localization of Cap43 may be controlled at least in part by phosphorylation. The Cap43 gene is localized in the nucleus of some cell types, but its protein structure has no apparent nuclear localization signals, suggesting that interactions with other protein(s) are required for its nuclear localization [11]. Cap43 protein interacts with a nucleocytoplasmic transport protein, heat-shock cognate protein 70, in mast cells [41,42]. Whether this interaction is required for the nuclear localization of Cap43 remains unclear. In cervical adenocarcinoma, Cap43 was localized primarily in tumor cell membranes. The reasons for this localization pattern are unknown. Further studies are

required to determine the function of this membrane-associated Cap43.

Our study had several limitations. We studied only patients with early-stage cervical adenocarcinoma treated by surgery. Because of the selection bias, the results cannot be directly extrapolated to larger populations of women with cervical adenocarcinoma (e.g., women with stage III or IV cervical adenocarcinoma). Cervical adenocarcinoma continues to have unfavorable outcomes. Assessment of Cap43 expression in cervical adenocarcinomas may provide a useful biomarker for the prediction of outcomes, independently of conventional clinical variables.

In conclusion, our study demonstrated, for the first time to our knowledge, that high expression of Cap43 in patients with cervical adenocarcinoma is associated with tumor angiogenesis and poor outcomes. Our findings remain preliminary and should be confirmed in prospective clinical trials. Moreover, further basic research is required to identify the pathways by which Cap43 protein modulates the malignant characteristics of tumors and to delineate the mechanisms underlying tumor progression in cervical adenocarcinoma.

Disclosure/conflict of interest

The authors declare no potential conflict of interest.

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