Fig. 2. Expression of the Cyclin D2 gene in ovarian cancer cell lines and normal ovarian tissue. Two independent reverse transcription–polymerase chain reactions were carried out for each sample, and the ratio of Cyclin D2: β-actin was calculated and normalized with the level of normal ovarian tissue. Methylation status is indicated in the same way as in Fig. 1.
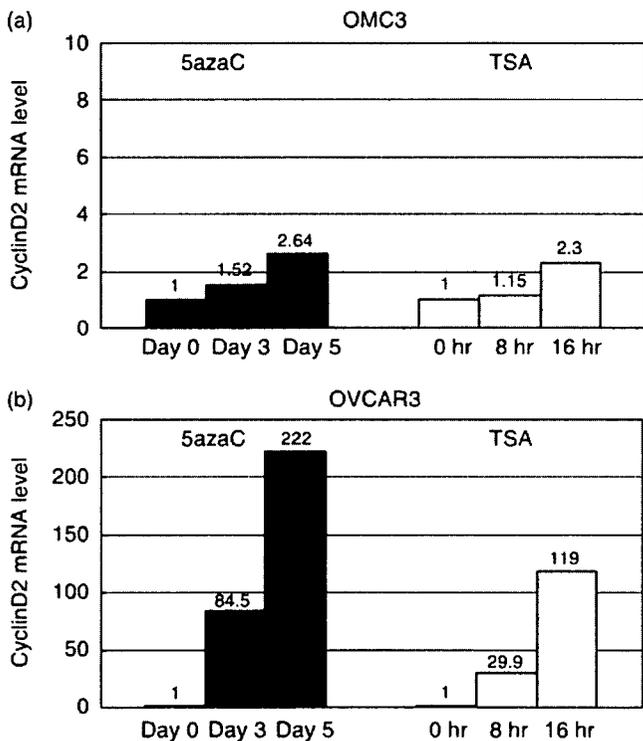

Fig. 3. Expression level of the Cyclin D2 gene as determined by quantitative reverse transcription–polymerase chain reaction in OMC3 and OVCAR3 cells following treatment with (a) 5-aza-2'-deoxycitidine (5azaC) or (b) trichostatin A (TSA). The ratio of Cyclin D2 : β-actin was calculated and normalized with the level before treatment.
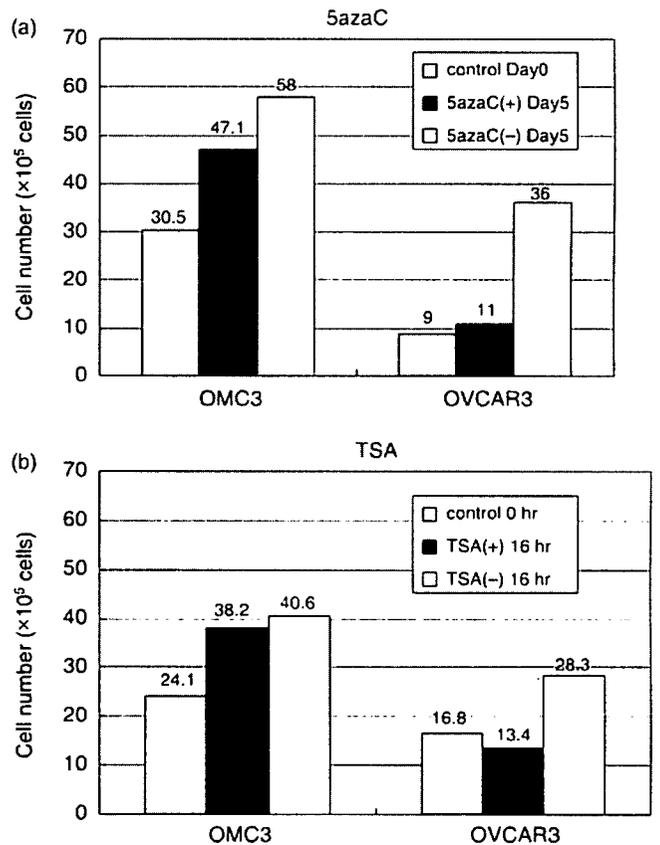

Fig. 4. Cell number of OMC and OVCAR3 cells following treatment with (a) 5-aza-2'-deoxycitidine (5azaC) or (b) trichostatin A (TSA). *Control treatment with medium alone.

There was no association between methylation status and age, performance status, histological type, histological grade or Ki-67 labeling index

The results of the univariate analysis of prognostic significance for each variable with respect to survival are summarized in Tables 2 and 3. Of the clinicopathological parameters evaluated, performance status, stage, histological grade and residual

tumor size were significantly associated with disease-free and overall survival. The methylation status of Cyclin D2 was significantly associated with disease-free survival; the cases with methylation had significantly worse rates of disease-free survival than those without methylation (Fig. 6; $P = 0.021$). With
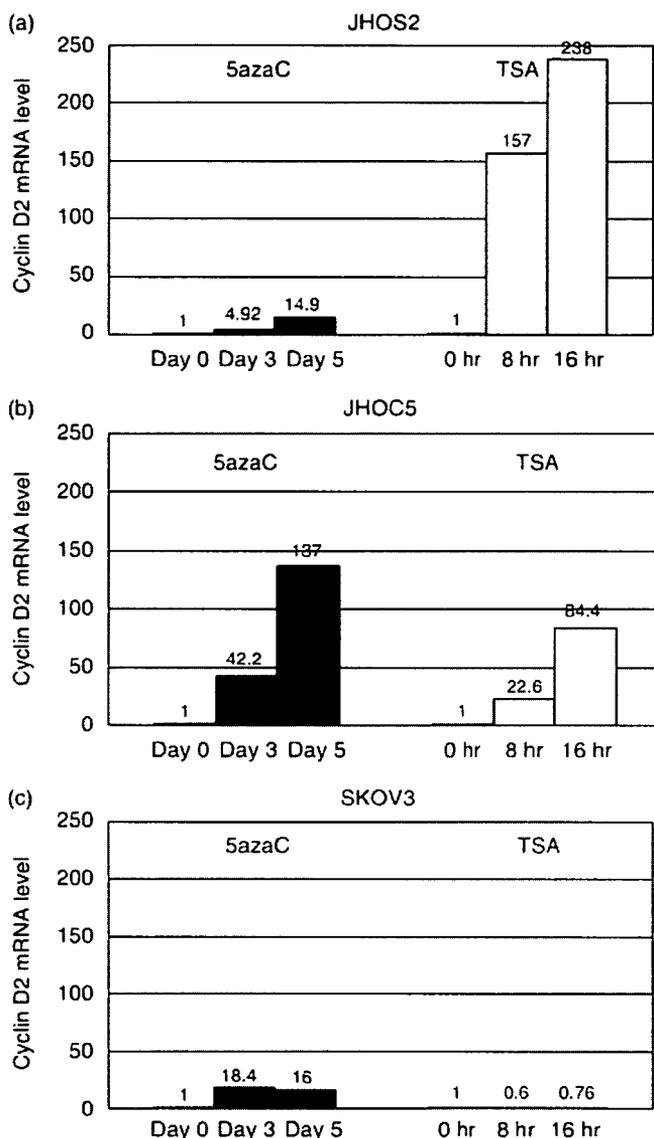
(a) JHOS2

5azaC TSA

238

157

1 4.92 14.9 1

Day 0 Day 3 Day 5   0 hr 8 hr 16 hr

(b) JHOC5

5azaC TSA

137

42.2

22.6 84.4

1 1

Day 0 Day 3 Day 5   0 hr 8 hr 16 hr

(c) SKOV3

5azaC TSA

18.4 16

1 1 0.6 0.76

Day 0 Day 3 Day 5   0 hr 8 hr 16 hr

Fig. 5. Expression level of the Cyclin D2 gene as determined by quantitative reverse transcription–polymerase chain reaction in (a) JHOS2, (b) JHOC5 and (c) SKOV3 cells following treatment with 5-aza-2'-deoxycitidine (5azaC) or trichostatin A (TSA). The ratio of Cyclin D2 : β-actin was calculated and normalized with the level before treatment.

regard to overall survival, methylated cases had a worse prognosis than unmethylated cases, but the difference was not significant (Fig. 7; $P = 0.063$). In multivariate analysis, methylation status of cyclin D2 turned out not to be an independent pro - gnostic factor (data not shown).

Discussion

Aberrant promoter methylation is found in many types of human cancer and is a common mechanism for transcriptional inactivation of various genes, including tumor suppressor genes, DNA repair genes, cell cycle regulatory genes and apoptosis-related genes. In the present study, we determined the Cyclin D2 promoter methylation status of several ovarian cancer cell lines and ovarian cancer surgical specimens, measured the levels of Cyclin D2 gene expression in ovarian cancer cell lines and

Table 2. Univariate analysis of disease-free survival

Variable	P-value
Cyclin D2 methylation status	0.0212
Age	0.6657
Performance status	<0.0001
FigO stage	0.0001
Histological type	0.4709
Grade	0.1332
Residual tumor	0.0008

Table 3. Univariate analysis of overall survival

Variable	P-value
Cyclin D2 methylation status	0.0625
Age	0.4195
Performance status	0.0003
FigO stage	0.0003
Histological type	0.0637
Grade	0.1983
Residual tumor	0.0016

Unmethylated cyclin D2

$P = 0.021$

Methylated cyclin D2
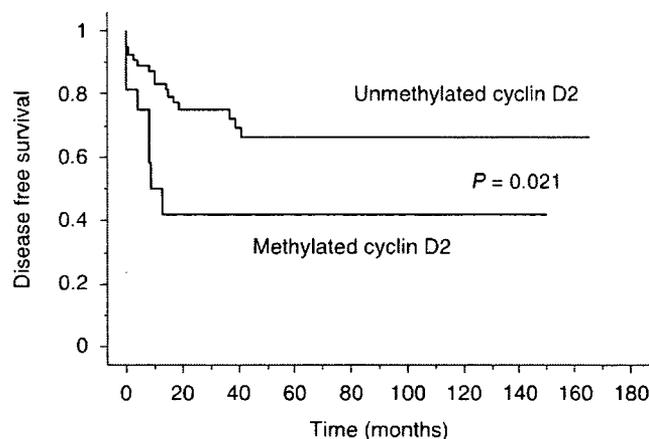
Fig. 6. Association between Cyclin D2 promoter methylation status and disease-free survival in patients with epithelial ovarian cancer.

Unmethylated cyclin D2
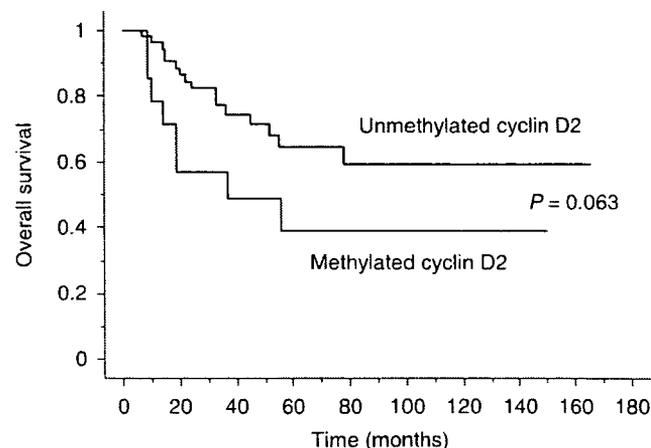
$P = 0.063$

Methylated cyclin D2

Fig. 7. Association between Cyclin D2 promoter methylation status and overall survival in patients with epithelial ovarian cancer.

linked the methylation status of the Cyclin D2 promoter to various clinical and pathological variables in ovarian cancer patients.

From MSP and quantitative RT-PCR analysis, there was a trend towards a reduction in gene expression in the presence of hypermethylation; however, this association was not significant, and it was suggested that expression of the Cyclin D2 gene in ovarian cancer cell lines, as a whole, was considerably low in comparison with that in normal ovarian tissue. There was an increase in Cyclin D2 gene expression following the 5azaC treatment of cell lines with promoter methylation of the Cyclin D2 gene in MSP. However, TSA or 5azaC treatment of the cell lines without methylation in MSP resulted in re-expression of the Cyclin D2 gene. Together with these findings, it is suggested that some epigenetic changes, including promoter methylation or histone deacetylation, might contribute to silencing of the Cyclin D2 gene in epithelial ovarian cancer cell lines. The re-expression by treatment with 5azaC in the unmethylated cell lines JHOS2 and JHOC5 suggests that the Cyclin D2 gene may be secondary re-expressed owing to activating other suppressed gene by promoter methylation with treatment of 5azaC, or there is a possibility that aberrant methylation did exist but in a different region of the Cyclin D2 promoter to that which we analyzed. Further investigation and data regarding the acetylation status of histones, a different DNA methylation analysis to decipher the MSP results, and DNA methylation of the transcription factor of Cyclin D2 are needed to supplement our hypothesis.

Epithelial ovarian cancer cell growth following treatment with 5azaC or TSA was suppressed in OMC3 and OVCAR3 cell lines. Treatment with these chemical agents resulted in inhibition of cell growth as well as re-expression of the Cyclin D2 gene. However, another tumor suppressor gene was also re-expressed by these treatments, and these chemicals could have cell toxicity in itself[26–28]. The present data suggests that 5azaC and TSA could be therapeutic agents targeting epigenetic changes in epithelial ovarian cancer, and epigenetic gene silencing of the Cyclin D2 gene could used as a marker of tumor growth.

The D-type cyclins are early checkpoint regulators at the G_1 phase of the cell cycle. Although well known for their proliferation-promoting activity, the D-type cyclins also have growth-inhibitory effects[14]. Thus, decreased expression of Cyclin D2 could result in abnormal cell proliferation and contribute to malignant transformation. Indeed, Cyclin D2 gene silencing secondary to DNA promoter methylation has been demonstrated in several human cancers[15–17,29]. Cyclin D2 promoter hypermethylation has also been detected in nearly half of breast cancers and is associated with gene silencing. Cyclin D2 hypermethylation has also been demonstrated in small cell and non-small cell lung

cancer tumor tissues and cell lines,[17] and in approximately half of gastric cancer specimens.[16] In the present study, 22.5% of the surgical specimens and 41.7% of the cell lines had aberrant Cyclin D2 promoter hypermethylation. Our results, though somewhat higher than what has been reported for ovarian granulosa cell tumors,[10] are similar to the percentages seen in several other cancers. Howver, some reports say that aberrant methylation of the Cyclin D2 promoter is an early event in tumorigenesis, as is suggested by its presence in ductal carcinoma in situ in breast cancer and its absence in normal ducts;[15,18,29] however, this epigenetic change was associated with advanced ovarian cancer in the present study. Our results suggest that aberrant methylation of this gene could be related to tumor progression rather than tumorigenesis of epithelial ovarian cancer.

A number of biological tumor variables, such as DNA ploidy, steroid hormone receptor status and the expression of certain oncogenes, are associated with prognosis in epithelial ovarian cancer.[30–32] The promoter methylation status of several genes, such as 14-3-3 sigma, BRCA1, hMLH1 and TMS1, has been used to predict poor survival in epithelial ovarian cancer patients.[9,24,33–35] In the present study, Cyclin D2 promoter methylation was significantly associated with advanced stage, a larger residual tumor size and poor prognosis. Because there was a trend toward the repression of gene expression in the presence of promoter hypermethylation in ovarian cancer cell lines, we presume that Cyclin D2 gene silencing might occur in primary tissues with methylation, though the levels of the Cyclin D2 gene have not been analyzed in this study. These results suggest that the aberrant promoter methylation of Cyclin D2, or decreased expression of this gene caused by methylation, may be associated with aggressive biological characteristics, and may play a significant role in disease progression in epithelial ovarian cancer.

The contribution of Cyclin D2 to the pathophysiology of epithelial ovarian cancer is not known at a rudimentary level. Though numerous studies have classified it as an oncogene, our data and that of others strongly supports the hypothesis that it functions as a tumor suppressor gene. Further studies are needed to better clarify the relationship between Cyclin D2 gene expression level and its function as either an oncogene or a tumor suppressor. A deeper understanding of the role of D-type cyclins in ovarian cancer tumor biology could provide a foundation on which to base new diagnostic tests or molecular therapies.

Acknowledgment

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ORIGINAL ARTICLE

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Progression-free survival and overall survival of patients with clear cell carcinoma of the ovary treated with paclitaxel-carboplatin or irinotecan-cisplatin: retrospective analysis

Abstract

Background. Irinotecan hydrochloride, a topoisomerase I inhibitor, has been preliminarily recognized as an effective agent against clear cell carcinoma of the ovary (CCC), but there are few clinical data. Our aim was to compare progression-free survival (PFS) between patients treated with irinotecan hydrochloride and cisplatin (CPT-P) and those with treated with paclitaxel and carboplatin (TC).

Methods. One hundred and seventeen patients at International Federation of Gynecology and Obstetrics (FIGO) stages Ic (ascites/malignant washing) – IV were identified by scanning the medical records of ten Japanese hospitals. After complete surgical staging procedures including lymphadenectomy, 35 patients received CPT-P and 82 patients received TC. The PFS and overall survival of the two groups were compared using the Kaplan-Meier method.

Results. There was no significant difference in median age, performance status, FIGO stage, rate of optimal cytoreduction, or follow-up period between the CPT-P and TC groups. Two-year and 5-year PFS was 48% and 40%, respectively, in the TC group and 55% and 55%, respectively, in the CPT-P group ($P = 0.31$). Multiple regression analysis revealed that only residual tumor was an independent prognostic factor for PFS ($P < 0.01$).

Conclusion. CPT-P showed a potential therapeutic effect, at least no less than that of TC therapy. Although there was no significant survival benefit in the present retrospective analysis, we recommend that the CPT-P regimen be evaluated in a larger, prospective, clinical trial.

Key words Ovarian cancer · Clear cell carcinoma · Irinotecan · Adjuvant chemotherapy · Paclitaxel · Progression-free survival

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Introduction

Clear cell carcinoma of the ovary (CCC) was initially termed "mesonephroma ovarii" by Schiller in 1939,[1] and in 1973 it was strictly defined by the World Health Organization as lesions characterized by clear cells growing in solid/tubular or glandular patterns, as well as hobnail cells.[2] Many publications have identified the distinctive behavior of CCC. The

most distinctive characteristics recognized are that patients with CCC had worse prognoses compared with those with other pathological types of epithelial ovarian carcinomas[3,4] and that CCC showed resistance to conventional platinum-based chemotherapy.[5-8]

Since the establishment of paclitaxel and carboplatin (TC) as the "gold standard" regimen for epithelial ovarian cancer,[9,10] the regimen has been widely used for all histological subtypes of ovarian tumors. But response in measurable CCC cases treated with TC was relatively low, ranging from 22% to 56%.[11-13] The survival benefit of the regimen is also controversial; one study showed superior survival benefit,[14] and another implied no survival benefit in either early or advanced cases.[15]

As irinotecan hydrochloride, a semisynthetic derivative of camptothecin, has been reported to have additive and synergistic effects in combination with cisplatin in vitro,[16-18] combination therapy with irinotecan and cisplatin (CPT-P) has been used clinically for patients with various solid tumors. Especially, a large clinical trial revealed that CPT-P had significant activity for extensive small-cell lung cancer.[19] Moreover, CPT-P has been reported to be effective in first-line and second-line chemotherapy for the treatment of CCC.[20-22] The aim of the present retrospective study was to compare the survival benefit of combination therapy with CPT-P with that of TC.

Patients and methods

A retrospective review of patients with CCC seen at ten Japanese hospitals from January 1, 1992 to December 31, 2003 was done. Of all the patients treated at these hospitals, the following patients were selected: (a) patients who underwent complete surgical staging procedures, including hysterectomy, bilateral salpingo-oophorectomy, peritoneal washing, omentectomy, pelvic lymphadenectomy, and para-aortic lymphadenectomy; (b) patients whose tumor specimens were confirmed as CCC by two pathologists in a central pathological review; (c) patients who were at Inter-

national Federation of Gynecology and Obstetrics (FIGO) stages Ic (ascites/malignant washing), II, III, and IV; (d) patients treated with six courses of combination chemotherapy using CPT-P, or six courses of TC; (e) age 75 years or less; (f) Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 2 or less; (g) pretreatment leukocyte count of 4000/mm^3 or more, platelet count of 100000/mm^3 or more, hemoglobin, 9.0g/dl or more, serum creatinine, less than 1.5mg/dl, creatinine clearance, 60ml/min or more, and GOT and GPT less than twice the upper limit of normal at the hospitals. The study was approved by the Ethics Committee at each hospital.

One cycle of the CPT-P regimen consisted of a drip infusion of 50–60mg/m^2 of cisplatin on day 1 and 50–60mg/m^2 of irinotecan on days 1, 8, and 15, and 1 week off and it was repeated every 4 weeks. The TC regimen consisted of a drip infusion of 175–180mg/m^2 of paclitaxel and carboplatin (AUC, 5–6).

The time to progression was defined as the interval from the date of primary surgery until the date of recurrence or tumor progression. Survival duration was determined as the time from the date of primary surgery until death or the date of last follow-up contact. The Kaplan-Meier method was used for the calculation of patient survival distribution. The significance of the survival distribution in each group was tested by a generalized Wilcoxon test and the log-rank test. The χ^2 test and Student's t-test for unpaired data were used for statistical analysis. A P value of less than 0.05 was considered statistically significant. Stat View software version 5.0 (SAS, Cary, NC, USA) was used to analyze the data.

Results

In all, 35 patients with the CPT-P regimen and 82 with the TC regimen were enrolled in the present retrospective study. The characteristics of the patients are outlined in Table 1. There was no significant difference in median age, performance status, FIGO stage, residual tumor diameter,
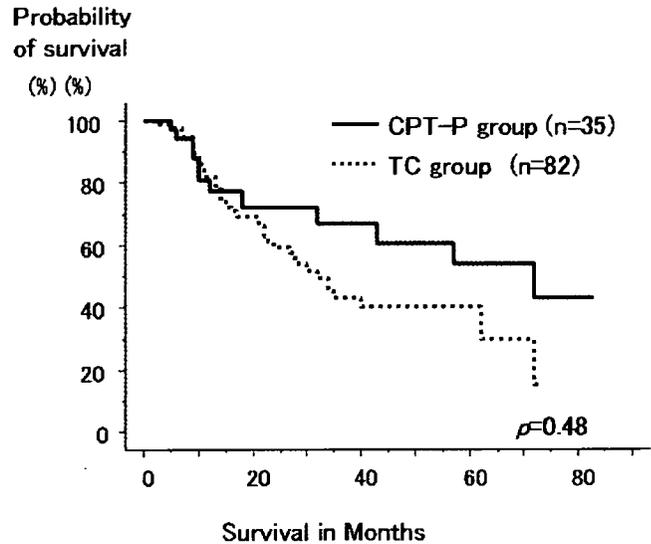
Table 1. Characteristics of the patients

	Irinotecan plus cisplatin	Paclitaxel plus carboplatin	P value
Patients (n)	35	82	
Median age, years (range)	52 (34–69) 54 (38–74)	0.79	
Performance status			0.12
0	18 (51%)	45 (55%)	
1, 2	17 (49%)	37 (45%)	
FIGO stage			0.94
Ic (Ascites/malignant washing)	13 (37%)	28 (34%)	
II	6 (17%)	15 (18%)	
III	13 (37%)	34 (41%)	
IV	3 (9%)	5 (6%)	
Residual tumor diameter			0.62
0 cm	23 (66%)	52 (63%)	
<1 cm	5 (14%)	8 (10%)	
>1 cm	7 (20%)	22 (27%)	
Follow-up period (months)			0.28
Median	17	21	
Range	5–83	3–73	

Table 2. Multiple regression survival analysis for stage Ic (ascites/malignant washing)-IV patients with clear cell carcinoma of the ovary

Variables (number of patients)	Hazard ratio	95% Confidence interval	P value
Age (years)			0.35
<50 (n = 38)	1		
>51 (n = 79)	1.33	0.73; 2.42	
Performance status			0.61
0 (n = 63)	1		
1, 2 (n = 54)	1.17	0.64; 2.14	
FIGO stage			0.16
Ic (Ascites/malignant washing), II (n = 62)	1		
III, IV (n = 55)	1.70	0.81; 3.56	
Residual tumor			<0.01
None (n = 75)	1		
<1 cm (n = 13)	2.54	1.16; 5.57	
>1 cm (n = 29)	3.17	1.35; 7.40	
Chemotherapy			0.21
Irinotecan and cisplatin (n = 35)	1		
Paclitaxel and carboplatin (n = 82)	1.55	0.79; 3.03	
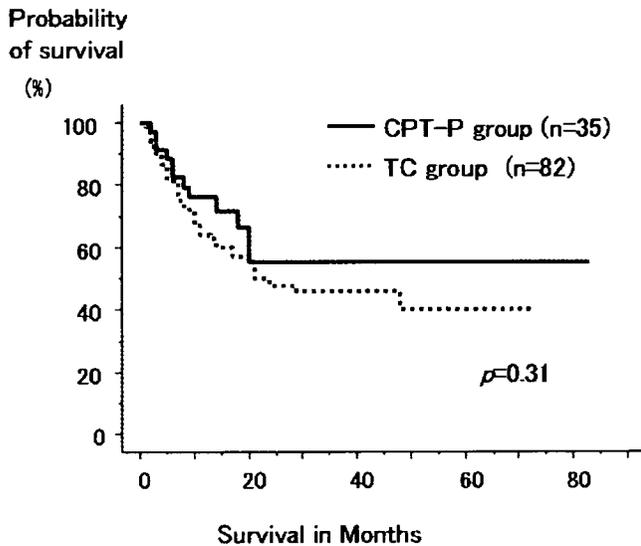
Fig. 1. Kaplan-Meier curves comparing the progression-free survival (PFS) of stage Ic (ascites/malignant washing) – IV patients according to adjuvant chemotherapy. The 2-year and 5-year PFS was 55% and 55%, respectively, in the irinotecan and cisplatin (CPT-P) group, and 48% and 40% in the paclitaxel and carboplatin (TC) group (P = 0.31)

Fig. 2. Kaplan-Meier curves comparing the overall survival of all the patients treated with the combination of irinotecan and cisplatin (CPT-P) and those treated with paclitaxel and carboplatin (TC; P = 0.48). The 2-year and 5-year overall survival was 72% and 54%, respectively, in the CPT-P group and 60% and 43% in the TC group

or follow-up period between the CPT-P group and the TC group. The median age was 52 years in the CPT-P group and 54 years in the TC group. The CPT-P group included 13 patients (37%) at stage Ic (ascites/malignant washing), 6 (17%) at stage II, 13 (37%) at stage III, and 3 (9%) at stage IV. In the TC group, 28 patients (34%) were at stage Ic, 15 (18%) at stage II, 34 (41%) at stage III, and 5 (6%) at stage IV. Optimal cytoreduction (residual tumor diameter <1 cm) with the initial surgery was achieved in 80% (28/35 patients) in the CPT-P group and 73% (60/82 patients) in the TC group. In patients with tumors at FIGO stages III and IV, the rate of optimal surgery was 56% (9/16 patients) in the CPT-P group and 46% (18/39 patients) in the TC group.

The median follow-up period was 17 months in the CPT-P group and 21 months in the TC group.

The 2-year and 5-year progression-free survival (PFS) rates were 55% and 55%, respectively, in the CPT-P group and 48% and 40% in the TC group (Fig. 1; P = 0.31). The 2-year and 5-year overall survival rates were 72% and 54%, respectively, in the CPT-P group and 60% and 43% in the TC group (Fig. 2; P = 0.48). Multiple regression analysis revealed that only residual tumor was an independent prognostic factor for PFS (P < 0.01; Table 2). Age, performance status, and FIGO stage were not significant prognostic factors. Additionally, chemotherapy was also not an independent factor for PFS in the CCC patients in the present

study (TC compared with CPT-P: hazard ratio, 1.55; 95% confidence interval, 0.79 to 3.03, $P = 0.21$).

Discussion

It has been well recognized that CCC has low sensitivity to conventional platinum-based chemotherapy.[3,4,7] But it is still uncertain which regimen would be the best candidate for CCC. Some reports have indicated a survival benefit of paclitaxel and platinum therapy in comparison with platinum-based chemotherapy.[10,12] A larger study implied that a combination with paclitaxel and platinum had almost the same impact on survival as conventional platinum-based chemotherapy in both early- and advanced-stage patients.[15]

The CPT-P regimen was initially introduced as a treatment for platinum-refractory ovarian cancer.[22] Since then, the regimen has been used for the treatment of CCC as first-line chemotherapy and has shown moderate activity against CCC.[20,21] The present study implies that the survival of patients treated with CPT-P might be improved compared with the survival of those treated with TC. However, our study was a limited retrospective study and failed to prove the superiority of the CPT-P regimen. The effectiveness of irinotecan as well as paclitaxel against CCC was also confirmed in vitro.[23] Combined with mitomycin C, irinotecan also showed higher activity than conventional platinum-based chemotherapy.[24] Chemotherapeutic regimens including irinotecan have been suggested to have a potential antitumor effect against CCC as first-line chemotherapy.

CCC has been reported to have distinct molecular characteristics compared with other histological subtypes. The overexpression of hepatocyte nuclear factor-1 beta[25] and that of ABCF2, a member of the ATP-binding cassette gene superfamily[26] were observed in CCC. These molecules might be another or additive target in the treatment of CCC.

Although there was no statistically significant difference in survivals between the CPT-P and TC regimens in our study, CPT-P was shown to have the same chemotherapeutic benefit in the survival of CCC patients as TC. Therefore, we recommend that the CPT-P regimen be tested in a large-scale prospective study.

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Original contribution

Expression of hypoxia-inducible factor 1α, hypoxia-inducible factor 2α, and von Hippel–Lindau protein in epithelial ovarian neoplasms and allelic loss of von Hippel-Lindau gene: nuclear expression of hypoxia-inducible factor 1α is an independent prognostic factor in ovarian carcinoma ☆

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Summary The hypoxia-inducible factor (HIF) is a transcriptional factor with important roles in tumor biology. To clarify the possible involvement of the HIF-α subunit and von Hippel–Lindau (VHL) protein in the development and progression of ovarian carcinoma, we analyzed the immunohistochemical expressions of HIF-1α, HIF-2α, and VHL in 107 cases of epithelial ovarian tumors. In addition, we examined loss of heterozygosity (LOH) at *VHL* gene loci. The frequency of the cytoplasmic expression of HIF-2α in carcinomas was higher than that in benign and borderline tumors ($P < .0001$). Furthermore, the nuclear expression of HIF-1α and the cytoplasmic expression of HIF-2α were significantly higher in tumors of FIGO (International Federation of Gynecology and Obstetrics) stages III and IV than in those of stages I and II. On the other hand, the cytoplasmic expression of HIF-1α did not show differences among histological malignancies. There was a positive correlation between nuclear HIF-1α expression and vascular endothelial growth factor ($\rho = 0.320, P < .001$). Although LOH at the *VHL* gene locus was frequent in ovarian carcinomas (24%), there is no significant correlation between LOH and loss of VHL expression. In 22 clear cell carcinomas, VHL expression showed a significantly

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negative correlation with the nuclear expression of HIF-1α ($\rho = -0.529$, $P = .0153$). The log-rank test showed that nuclear positive immunostaining for HIF-1α ($P = .002$) and cytoplasmic positive immunostaining for HIF-2α ($P = .0112$) in tumor cells are associated with poor prognosis of patients with ovarian carcinoma. Multivariate analysis also showed that the nuclear expression of HIF-1α is an independent prognostic factor. These results show that the HIF-α subunit represents an important biomarker in the evaluation of the prognosis of patients with ovarian carcinoma.

1. Introduction

Epithelial ovarian carcinoma is the leading cause of death from female genital malignancies, and more than half of patients are diagnosed at advanced stages with peritoneal dissemination [1]. Peritoneal dissemination is a metastatic process in which the cancer cells detach from the primary tumor, attach to the peritoneum, and grow at this site. Ovarian carcinoma cells leaving the primary tumor may therefore experience lower oxygen levels [2]. Recent attention has focused on the role that the surrounding microenvironment plays in the process of tumorigenesis as well as tumor progression and how it contributes to tumor biology [3,4]. We also reported that associations between microenvironmental hypoxia and aggressively invasive phenotypes are observed in ovarian carcinomas [5].

The hypoxia-inducible factor (HIF) is an α/β heterodimeric DNA binding complex and directs an extensive transcriptional response involving the induction of genes relevant to tumor progression, such as angiogenesis, glucose/energy metabolism, cellular growth, metastasis, and apoptosis [6,7]. The HIF-α subunit interacts with von Hippel–Lindau (VHL) protein and is degraded by ubiquitin-mediated proteolysis in the presence of oxygen. To date, 3 HIF-α isoforms have been reported, the best characterized being HIF-1α and HIF-2α, which are members of the basic helix-loop-helix/PAS domain protein family. It has been reported that the HIF system is upregulated by micro-environmental hypoxia and by genetic events in human malignancy [7]. HIF-1α and HIF-2α have different effects during embryonic development [8,9]. In vitro studies have also shown that the hypoxia response is critically dependent on the different isoforms in different tumor types [10-12]. In this study, we assessed the expressions of HIF-1α and HIF-2α in ovarian carcinomas and determined their associations with progression and overall outcome.

To clarify the possible involvement of HIF-1α, HIF-2α, VHL, and their mutual relationship in the development and progression of ovarian carcinoma, we analyzed the immunohistochemical expressions of HIF-1α, HIF-2α, VHL, vascular endothelial growth factor (VEGF), and CD34 in 107 cases of epithelial ovarian tumors. In addition, we examined loss of heterozygosity (LOH) at VHL gene loci. Finally, we analyzed correlation and prognostic differences according to the expressions of HIF-1α, HIF-2α, VHL, VEGF, and micro-vessel density (MVD) in patients with ovarian carcinoma.

2. Materials and methods

2.1. Patients and tissue samples

One hundred seven primary epithelial ovarian tumors were examined for immunohistochemistry. Seventy-two consecutive patients with ovarian carcinoma visited the Shinshu University Hospital between 1995 and 2003 and underwent surgery followed by cisplatin-based chemotherapy. The follow-up period ranged from 3 to 131 months (median, 52 months). Specimens were reviewed to confirm the histopathological diagnoses with the use of standard criteria [13]. Histologically, 18 of the 107 tumors were benign (7 serous and 11 mucinous cystadenomas), 17 were borderline (6 serous and 11 mucinous tumors), and 72 were carcinomas (26 serous, 7 mucinous, 17 endometrioid, and 22 clear cell adenocarcinomas). Of the 72 carcinomas, 39 were classified as stage I, 10 as stage II, 20 as stage III, and 4 as stage IV according to FIGO (International Federation of Gynecology and Obstetrics) classification. With regard to histological grade [14], of the carcinomas, 32 were G1, 30 were G2, and 10 were G3. These specimens were fixed in 10% phosphate-buffered formalin and embedded in paraffin wax. Serial 3-μm sections were cut for hematoxylin-eosin staining and immunohistochemistry. Each tissue was used with the approval of the ethics committee of the Shinshu University.

2.2. Immunohistochemistry

For HIF-1α immunostaining, a catalyzed signal amplification system (Dako, Carpinteria, CA) was used as described previously [5]. In brief, after deparaffinization and rehydration, the sections were treated with a target retrieval solution (Dako) at 95°C for 45 minutes. The primary antibody, mouse anti–HIF-1α monoclonal antibody (Novus Biologicals, Littleton, CO), was used at a dilution of 1:1000.

Immunohistochemical staining for HIF-2α was performed with the use of a Histofine Simple Stain MAX-PO kit (Nichirei, Tokyo, Japan). The sections were deparaffinized and then treated with 0.3% hydrogen peroxide and incubated with 10% normal mouse serum to block nonspecific binding. The primary antibody, anti–HIF-2α mouse monoclonal antibody (EP190b, Novus Biologicals), was used at a dilution of 1:2000, as described previously [15]. We confirmed the specificity of the anti–HIF-1α

monoclonal antibody and that of the anti–HIF-2α monoclonal antibody with the use of Western blotting using ovarian cancer cell lines cultured under normoxia and hypoxia (data not shown).

For VHL, VEGF, and CD34 immunostainings, the streptavidin-biotin-peroxidase complex method (Histofine SAB-PO kit, Nichirei) was used. After deparaffinization and rehydration, the sections were boiled in 0.01 mol/L of citrate buffer (pH 6.0) for 15 minutes in a microwave oven. The primary antibodies used were monoclonal anti-VHL antibody (Ig33; NeoMarkers, Fremont, CA) and polyclonal anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA), which were used at a dilution of 1:50-100. For the analysis of MVD, mouse monoclonal anti-CD34 antibody (QBEnd/10, Novocastra Laboratories Ltd, Newcastle, UK) was used at a dilution of 1:25.

After incubation with the primary antibody at 4°C overnight, the sections were washed in phosphate-buffered saline and incubated with biotinylated goat antimouse or antirabbit immunoglobulin G, treated with peroxidase-conjugated streptavidin, and then stained with diaminobenzidine and 0.15% hydrogen peroxidase. Counterstaining was performed with hematoxylin.

For the assessment of cytoplasmic staining, we separately evaluated the percentage of positive cells and staining intensity (negative, 0; weak, 1; moderate, 2; strong, 3) under standard light microscopy. We used cervical cancer tissue as a strongly positive control for HIF-1α and macrophage as that for HIF-2α, as reported previously [16,17]. Negative controls were performed by substituting the primary antibodies with nonimmune sera. Staining scores were calculated by multiplying the percentage of positive cells (0-100) by the staining intensity (0-3) and therefore ranged from 0 to 300. Immunostaining was evaluated by 2 independent observers (R. O. and A. H.) unaware of the patients or the tissue sites. The results of immunostaining were classified as negative (−) when the staining score was between 0 and 30, weakly positive (+) when the staining score was between 31 and 120, and strongly positive (++) when the staining score was between 121 and 300. Nuclear immunostaining was observed sporadically in the tumor cells. The cases were classified as positive (>5% of tumor cells with nuclear staining) or negative (<5% of tumor cells with nuclear staining). MVD was quantified with the use of slides with CD34 staining [18,19]. We observed all slides at low-power magnification to identify the areas with the highest number of vessels within the tumor, and we counted vessels in a ×200 field.

2.3. DNA preparation

For DNA preparation, 64 epithelial ovarian tumors with matching normal DNA were available, including 9 benign, 10 borderline, and 45 malignant tumors. Sections of 8-μm thickness were deparaffinized, rehydrated, and dried, after which the fields of interest were selected and micro-

dissected under a dissection microscope with the use of a 23-G needle [20]. The cells were digested for 16 to 24 hours at 55°C in a digestion buffer (2 mg/mL of proteinase K and 0.5% Tween 20) and then treated with phenol-chloroform to extract DNA.

2.4. LOH analysis

Two microsatellite markers, D3S1317 and D3S1539, were used for the analysis of LOH [21,22]. DNA from tumoral areas and that from normal areas were amplified by polymerase chain reaction (PCR) separately with Ready-To-Go PCR Beads (Amersham, Piscataway, NJ). The PCR conditions were denaturation at 95°C for 5 minutes, 40 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 40 seconds as well as a final extension of 72°C for 10 minutes. PCR products and microsatellite allele sizes were determined with the use of an ABI 377 sequencing instrument (Perkin-Elmer, Waltham, MA). Genotyper 2000 software (Perkin-Elmer) was used to compare the relative intensities of the 2 alleles and determine LOH according to the manufacturer's criteria; the presence of LOH was strongly suspected if the ratio of peak heights on the electropherogram corresponding to the tumor and normal alleles was lower than 0.67 or greater than 1.35. A case was considered to be positive for LOH if at least 1 of the 3 markers showed a pattern of allelic loss, as reported previously [20].

2.5. Statistical analysis

Fisher's exact test, Kruskal-Wallis test, Scheffe test, and Mann-Whitney U test were used to assess the differences in immunoreactivity and LOH of VHL according to histological type, histological grade, and FIGO stage. Spearman's rank correlation was used to determine whether there was a positive or negative correlation. Differences were considered significant if the P value was lower than .05.

The log-rank test and the Cox proportional hazards model were used to evaluate significant predictors of survival. The prognostic factors used in the survival analysis were as follows: FIGO stage (I and II versus III and IV); histological grade (G1 versus G2 and G3); and results of immunostainings for cytoplasmic HIF-1α and HIF-2α (positive [+ and ++] versus negative), nuclear HIF-1α and HIF-2α (positive versus negative), as well as VHL (positive versus negative). The log-rank test and Cox univariate analysis were first performed for each of the factors. For multivariate analysis, overall survival was then analyzed by the stepwise regression model with the use of variables that exhibited significance by the Cox univariate analysis. A P value lower than .05 was considered significant. Cumulative survival was also analyzed by the Kaplan-Meier method. These analyses were made with the use of the StatView system (Abacus, Berkeley, CA) and SPSS version 14 (SPSS Inc, Chicago, IL).

3. Results

3.1. Immunohistochemistry of HIF-1α, HIF-2α, and VHL in epithelial ovarian tumors

3.1.1. Expression of HIF-1α

Representative profiles of immunostainings for HIF-1α, HIF-2α, and VHL are shown in Fig. 1. The results of HIF-1α immunostaining in epithelial ovarian neoplasms are shown in Table 1. Although HIF-1α staining was mainly observed in the cytoplasm, nuclear staining was sporadically observed in ovarian carcinoma cells (Fig. 1A-D). The cytoplasmic expression of HIF-1α in ovarian epithelial tumors did not show a significant difference among the histological malignancies (Table 1). With regard to cytoplasmic staining of HIF-1α, we evaluated staining intensity and percentage of positive cells separately, and the results also showed that differences among benign, borderline, and malignant tumors were not significant.

For the nuclear expression of HIF-1α, 2 (11%) of the 18 benign tumors, 2 (12%) of the 17 borderline tumors, and 24 of the 72 ovarian carcinomas (33%) were positive. The frequency of the nuclear expression of HIF-1α in carcinomas was higher than that of benign and borderline tumors, but it was not significant (Table 1). With regard to FIGO stage classification, nuclear immunostaining for HIF-1α was observed in 12 of the 48 cases of stages I and II (25%) and in 12 of the 24 cases of stages III and IV (50%). HIF-1α nuclear expression was significantly higher in tumors of FIGO stages III and IV than in those of stages I and II (P =
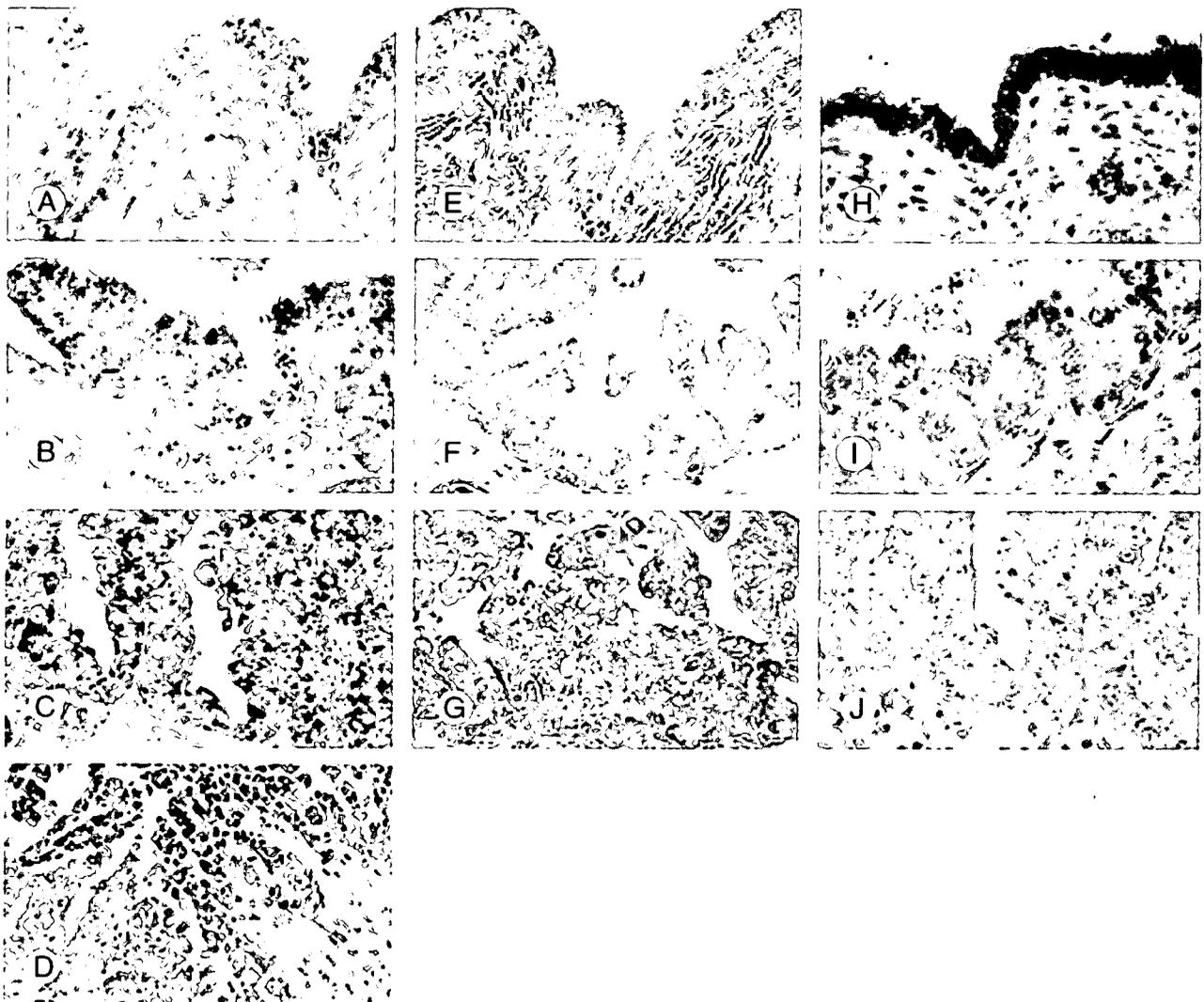
Fig. 1   Immunohistochemical staining of HIF-1α (A-D), HIF-2α (E-G), and VHL (H-J) in various epithelial ovarian tumors. A, Serous cystadenoma. B, Serous borderline tumor. C, Serous adenocarcinoma. D, Serous adenocarcinoma for HIF-1α. E, Serous cystadenoma. F, Serous borderline tumor. G, Serous adenocarcinoma for HIF-2α. H, Serous cystadenoma. I, Serous borderline tumor. J, Serous adenocarcinoma for VHL (original magnification ×400).

Table 1 Immunohistochemical expression of HIF-1α in epithelial ovarian neoplasms

	Total no. of cases	Cytoplasmic staining (n)			Nuclear staining (n)	
		−	+	++	−	+
Benign cystadenomas	18	4 (22%)	6 (33%)	8 (44%)	16 (89%)	2 (11%)
Serous	7	2	2	3	7	0
Mucinous	11	2	4	5	9	2
Borderline tumors	17	6 (35%)	7 (41%)	4 (24%)	15 (88%)	2 (12%)
Serous	6	0	3	3	4	2
Mucinous	11	6	4	1	11	0
Carcinomas	72	15 (21%)	28 (39%)	29 (40%)	48 (67%)	24 (33%)
FIGO stage						
I	38	7	13	18	27	11*
II	10	4	4	2	9	1
III	20	2	10	8	9	11
IV	4	2	1	1	3	1
Histological type						
Serous	26	6	9	11	17	9
Mucinous	7	3	3	1	6	1
Endometrioid	17	3	6	8	14	3
Clear cell	22	3	10	9	11	11
Histological grade						
G1	32	8	10	14	25	7
G2	30	5	14	11	17	13
G3	10	2	4	4	6	4

NOTE. Cytoplasmic immunostaining was estimated as follows: −, staining score was between 0 and 30; +, staining score was between 31 and 120; ++, staining score was between 121 and 300. Nuclear immunostaining was classified as follows: −, <5% of tumor cells with nuclear staining; +, >5% of tumor cells with nuclear staining.

* $P = .0338$.

.0338). Irrespective of histology, however, carcinoma cells with nuclear HIF-1α immunoreactivity were observed frequently in the tip of the papillary projection of the tumor (Fig. 1D) or in the vicinity of the necrotic area.

3.1.2. Expression of HIF-2α

The results of HIF-2α immunostaining in epithelial ovarian neoplasms are shown in Table 2. HIF-2α protein was mainly expressed in the cytoplasm of the tumor cells (Fig. 1E-G). In some ovarian carcinomas, a subset of cells morphologically identified as macrophages showed abundant cytoplasmic HIF-2α immunoreactivity (Fig. 2A) near the tumor or infiltrating the tumor stroma. These cells were confirmed as macrophages by examining serial sections stained for HIF-2α and CD68, a cell surface antigen specific to macrophages (Fig. 2B). For evaluation of HIF-2α staining in ovarian epithelial tumors, we excluded the expression of HIF-2α in macrophages.

All of the 18 benign cystadenomas were negative in the cytoplasmic staining score of HIF-2α. Of the 17 borderline tumors, 13 (76%) were negative and 4 (24%) were weakly positive for HIF-2α. Of the 72 carcinomas, 18 (25%) were negative, 25 (35%) were weakly positive, and 29 (40%) were strongly positive for HIF-2α. Accordingly, cytoplasmic HIF-2α expression was significantly higher in ovarian carcinomas than in benign and borderline tumors ($P < .0001$; Table 2). With regard to FIGO stage classification, negative immunostaining for

HIF-2α was observed in 16 of the 48 cases of stages I and II (33%) but in only 2 of the 24 cases of stages III and IV (8%). HIF-2α protein expression was significantly higher in tumors of FIGO stages III and IV than in those of stages I and II ($P = .0188$). With regard to cytoplasmic staining of HIF-2α, we also evaluated staining intensity and percentage of positive cells separately, and the results also showed that differences among benign, borderline, and malignant tumors were significant either in the staining intensity or in the number of positive cells. Significant differences between cases of FIGO stages I and II and those of FIGO stages III and IV were also noted in the number of positive cells.

Nuclear expression of HIF-2α was less frequently observed in the tumor cells. All of the benign cystadenomas and borderline tumors were negative for nuclear HIF-2α expression. Of the 72 carcinomas, 17 (24%) were positive for nuclear HIF-2α. The frequency of nuclear HIF-2α expression was significantly higher in ovarian carcinomas than in benign and borderline tumors ($P = .0074$; Table 2). Among the carcinomas, there was no difference in nuclear HIF-2α expression according to histological type, FIGO stage, and grade.

3.1.3. Expression of VHL protein

The results of VHL immunostaining in epithelial ovarian neoplasms are shown in Table 3. The immunohistochemical expression for VHL was observed in the cytoplasm of the

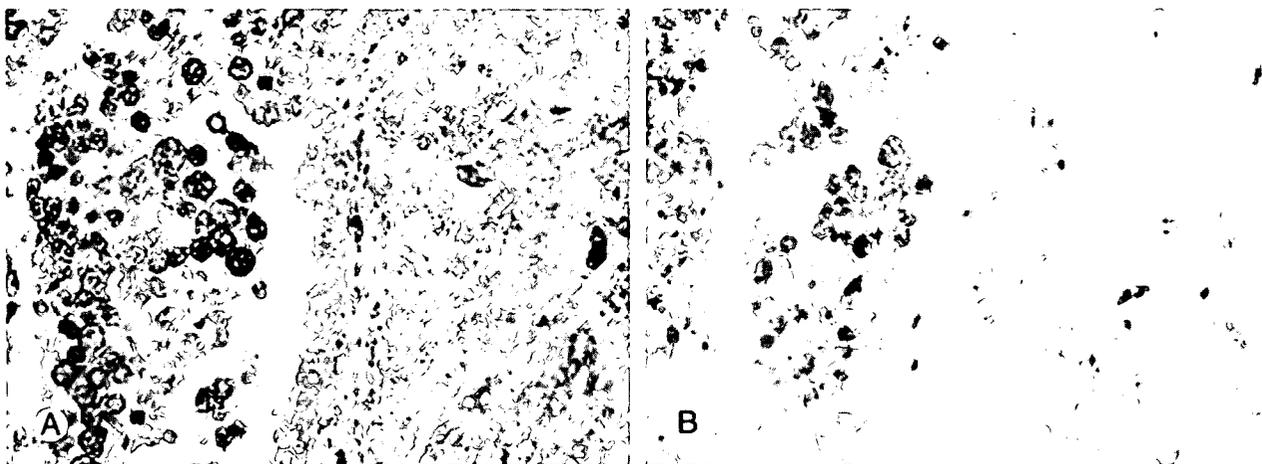**Table 2**   Immunohistochemical expression of HIF-2α in epithelial ovarian neoplasms

	Total no. of cases	Cytoplasmic staining (n)			Nuclear staining (n)	
		−	+	++	−	+
Benign cystadenomas	18	18 (100%)	0 (0%)	0**** (0%)	18 (100%)	0*** (0%)
Serous	7	7	0	0	7	0
Mucinous	11	11	0	0	11	0
Borderline tumors	17	13 (76%)	4 (24%)	0**** (0%)	17 (100%)	0*** (0%)
Serous	6	4	2	0	6	0
Mucinous	11	9	2	0	11	0
Carcinomas	72	18 (25%)	25 (35%)	29**** (40%)	55 (76%)	17*** (24%)
FIGO stage						
I	38	16	10	12**	28	10
II	10	0	3	7	10	0
III	20	2	10	8	13	7
V	4	0	2	2	4	0
Histological type						
Serous	26	2	15	9*	21	5
Mucinous	7	3	0	4	6	1
Endometrioid	17	5	4	8	15	2
Clear cell	22	8	6	8	13	9
Histological grade						
G1	32	12	11	9	24	8
G2	30	6	11	13	23	7
G3	10	0	3	7	8	2

* $P = .0344$.
** $P = .0188$.
*** $P = .0074$.
**** $P < .0001$.

tumor and normal stromal cells (Fig. 1H-J). Although reduced expression of VHL was frequently observed in ovarian carcinomas, the expression of VHL in ovarian epithelial tumors did not show a significant difference (Table 3). Among the carcinomas, there was no difference in VHL expression according to histological type, FIGO stage, and grade.

3.2. LOH at the VHL locus in various ovarian tumors

LOH was not detected in either the 9 benign tumors or the 10 borderline tumors examined. In carcinomas, LOH was more frequently detected as compared with benign and borderline tumors, being present in 11 (24%) of the 45 examined. There was no difference in clinicopathological

Fig. 2   Serial sections for the immunolocalizations of HIF-2α (A) and CD68 (B) in serous adenocarcinomas. A, Positive expression of HIF-2α in tumor cells and macrophages (original magnification ×250). B, Serial section showing CD68-positive macrophages (original magnification ×250).

Table 3 Immunohistochemical cytoplasmic expression of VHL in epithelial ovarian neoplasms

	Total no. of cases	Cytoplasmic staining (n)		
		−	+	++
Benign cystadenomas	18	4	8	6
Serous	7	0	2	5
Mucinous	11	4	6	1
Borderline tumors	17	7	8	2
Serous	6	3	2	1
Mucinous	11	4	6	1
Carcinomas	72	24	42	6
FIGO stage				
I	38	15	19	4
II	10	0	10	0
III	20	7	11	2
V	4	2	2	0
Histological type				
Serous	26	9	15	2
Mucinous	7	3	3	1
Endometrioid	17	3	13	1
Clear cell	22	9	11	2
Histological grade				
G1	32	10	18	4
G2	30	10	18	2
G3	10	4	6	0

characteristics and VHL protein expression between LOH-positive and LOH-negative carcinomas (Table 4).

3.3. Correlations among the expressions of HIF-1α, HIF-2α, VHL, and MVD

Correlations among the expressions of HIF-1α, HIF-2α, VHL, and MVD are shown in Table 5. The expression of nuclear HIF-1α showed a positive correlation with VEGF ($\rho = 0.320$, $P < .001$) in all ovarian carcinomas. Although there was no significant correlation between HIF-1α and VHL expressions ($\rho = -0.106$, $P = .372$) in all ovarian carcinomas, the expression of HIF-1α showed a significantly negative correlation with VHL ($\rho = -0.529$, $P = .0153$) in 22 clear cell carcinomas.

3.4. Topological correlation between HIF-1α, HIF-2α, and VHL

Closer observation with the use of serial sections on the immunoreactivity for HIF-1α, HIF-2α, and VHL disclosed that tumor cells with HIF-1α expression were associated with reduced expression of VHL as compared with the surrounding tumor cells that were negative for HIF-1α (Fig. 3). Such reduced expression of VHL along with HIF-1α expression was observed in 17 of the 72 cases (24%). Reduced expression of VHL along with cytoplasmic HIF-1α expression was frequently observed especially in clear cell adenocarcinoma cases (41%).

We also evaluated cytoplasmic staining of HIF-2α together with reduced VHL immunoreactivity. Such reduced

expression of VHL along with cytoplasmic HIF-2α expression was observed in only 14 of the 72 cases (19%).

3.5. Patient survival according to HIF-1α, HIF-2α, VHL, and MVD

All 17 patients with borderline tumors were alive at the last follow-up. Of the 72 patients with carcinoma, 32 died of their disease and the remaining 40 were alive. The prognosis was significantly poorer in patients with advanced FIGO stages (overall survival; 63.1 ± 36.1 months for stages I and II versus 29.8 ± 24.5 months for stages III and IV, $P < .0001$) and in those with higher-grade tumors (64.1 ± 32.0 months for G1 versus 43.2 ± 37.0 months for G2 and G3, $P = .0010$). In the 72 patients with ovarian carcinoma, the prognostic significance of HIF-1α, HIF-2α, VHL, as well as VEGF immunostainings and that of MVD were analyzed with the use of the Kaplan-Meier method. The results obtained by log-rank test showed that the prognosis was statistically significantly poorer in patients with positive immunostaining for nuclear HIF-1α (37.1 ± 32.8 months for positive staining versus 59.5 ± 35.8 months for negative staining, $P = .0022$; Fig. 4A), although immunostaining for cytoplasmic HIF-1α was not significant. Patients with a positive HIF-2α expression showed poorer survival as compared with those who had a negative expression (45.9 ± 35.1 months for weakly and strongly positive expressions versus 70.3 ± 34.0 months for negative expression, $P = .0112$; Fig. 4B). Univariate analysis with the use of the Cox proportional hazard model revealed the same tendency as that obtained with the use of the log-rank test. Multivariate analysis for FIGO stage, histological grade, and immunostainings for HIF-1α, HIF-2α, and VHL in ovarian cancer cases also showed that the nuclear expression of HIF-

Table 4 LOH at VHL locus

	Total no. of cases	LOH at VHL locus (n)	
		−	+
Carcinomas	45	34 (73%)	11 (28%)
FIGO stage			
I	20	17	3
II	7	4	3
III	14	11	3
IV	4	2	2
Histological type			
Serous	18	11	7
Endometrioid	14	11	3
Clear cell	13	12	1
Histological grade			
G1	19	15	4
G2	18	13	5
G3	8	6	2
VHL expression			
−	17	12	5
+	26	21	5
++	2	1	1

Table 5 Spearman's correlations between immunostainings for HIF-1α, HIF-2α, VHL, VEGF, and MVD

	HIF-1α	HIF-1α(N)	HIF-2α	VHL	VEGF	MVD
HIF-1α		0.244*	0.063	−0.082	0.179	0.004
HIF-1α(N)			0.176	0.039	0.336**	−0.009
HIF-2α				0.188	0.243	−0.052
VHL					0.171	0.184
VEGF						0.092
MVD						

Abbreviation: HIF-1α(N), nuclear staining of HIF-1α.
* Correlation is significant at the .05 level.
** Correlation is significant at the .01 level.

1α was an independent prognostic factor ($P = .007$) but that the cytoplasmic expression of HIF-2α was not ($P = .13$).

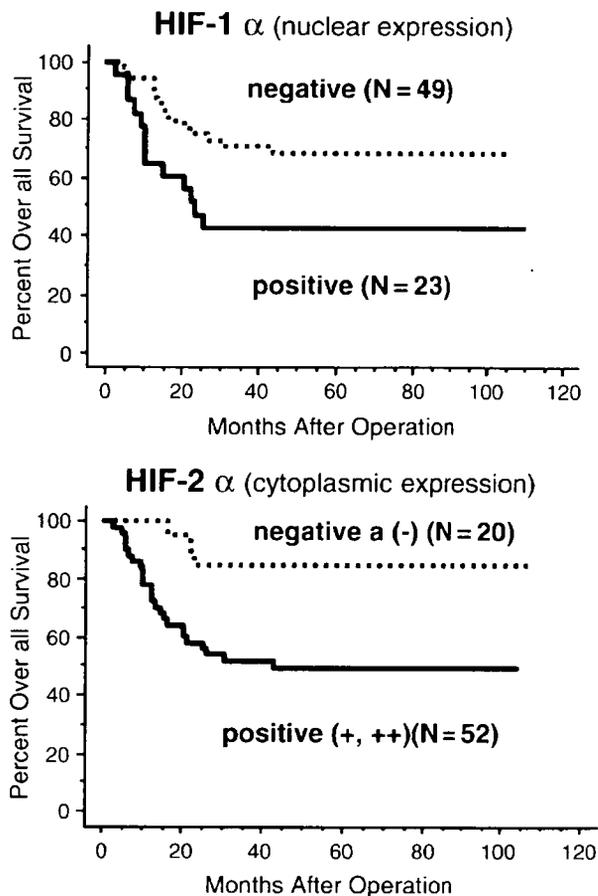## 4. Discussion

In this study, we investigated the immunohistochemical expressions and localizations of HIF-1α, HIF-2α, and VHL in ovarian epithelial neoplasms. The frequency of cytoplasmic expression of HIF-2α in carcinomas was higher than that in benign and borderline tumors. In addition, the nuclear expression of HIF-1α and the cytoplasmic expression of HIF-2α were significantly higher in tumors of FIGO stages III and IV than in those of FIGO stages I and II. On the other hand, cytoplasmic expression of HIF-1α did not show differences among histological malignancies
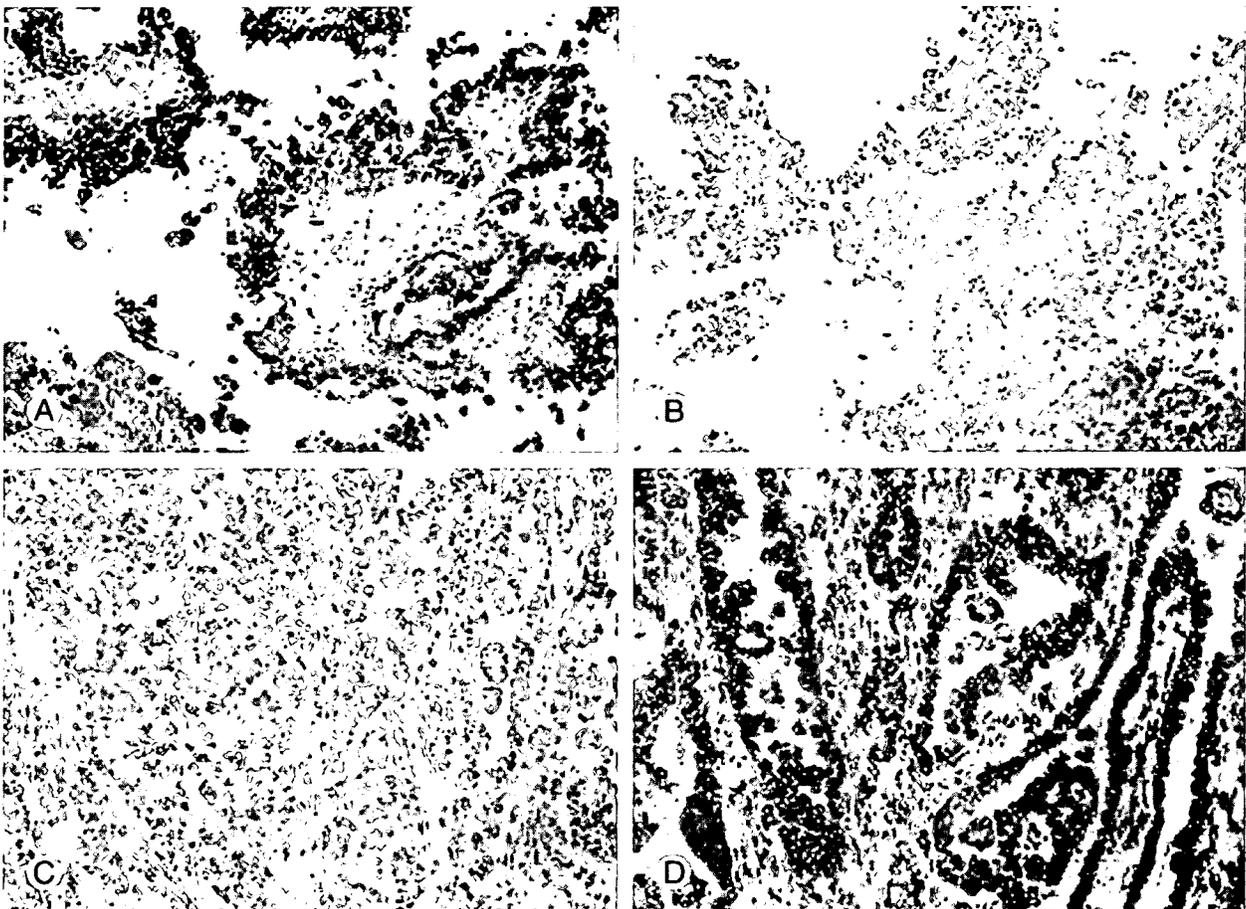
Fig. 3 Topological correlation between HIF-1α and VHL. In serial sections for the immunolocalizations of HIF-1α and VHL, the tumor cells with cytoplasmic expression of HIF-1α (A and C) are associated with reduced or loss of VHL expression (B and D), compared with the surrounding tumor cells negative for HIF-1α (original magnification ×100).

HIF-1 α (nuclear expression)

negative (N = 49)

positive (N = 23)

HIF-2 α (cytoplasmic expression)

negative a (-) (N = 20)

positive (+, ++)(N = 52)

Fig. 4 Overall survival of patients with ovarian carcinoma according to the expressions of HIF-1α (A) and HIF-2α (B). Kaplan-Meier analysis showed that the prognosis was significantly poorer in patients with positive nuclear immunostaining for HIF-1α. Patients with positive HIF-2α showed significantly poorer survival as compared with those with negative HIF-2α.

and was noted equally in early and advanced tumor stages. It has been reported that increased levels of HIF-1α are found in human cancers [7]. In addition, overexpression of HIF-2α has been reported in endometrial carcinomas [23], bladder tumors [24], lung cancers [25], and colorectal cancers [15]. To our knowledge, this is the first report on the expression of HIF-2α in epithelial ovarian tumors. Our findings suggest that the nuclear expression of HIF-1α and the higher cytoplasmic expression of HIF-2α may be hallmarks of malignancy and associated with the progression of ovarian carcinoma.

The results obtained by the log-rank test showed that the nuclear positive immunostaining for HIF-1α and the strong expression of cytoplasmic HIF-2α in tumor cells are associated with poor prognosis in patients with ovarian carcinoma. Multivariate analysis also showed that the nuclear expression of HIF-1α was an independent prognostic factor. HIF-1α is known to be translocated into the nucleus under hypoxia, where it is involved in gene transcription

[6,26]. Accordingly, the unfavorable prognosis of patients may be ascribed to the presence of hypoxic conditions. It has been reported that expression of HIF-1α had a significant impact and may be predictive of responsiveness to adjuvant therapy and radiotherapy in human malignancy [27-29]. On the other hand, in lung and colorectal carcinomas, HIF-1α had no impact on patient survival, but overexpression of HIF-2α was a prognostic indicator [15,25]. These findings suggest that different HIF-α isoforms may have distinct roles in different tumor types. In ovarian carcinomas, one study showed that HIF-1α overexpression alone was not a prognostic indicator and became a strong prognostic marker in combination with functional p53 protein [16]; however, that report did not describe the cellular localization of HIF-1α staining in ovarian carcinomas. From our observations, nuclear HIF-1α might represent an important biological marker in the evaluation of the prognosis of patients with ovarian carcinoma.

In this study, HIF-2α was detected predominantly in the cytoplasm of tumor cells. This is compatible with HIF-2α being detected predominantly in the cytoplasm of tumor cells and macrophages [17,30]. Although the biological significance of HIF-2 cytoplasmic expression is unknown, HIF-2α might be rapidly shuttled out of the nucleus and accumulate in the cytoplasm. Another possible explanation is that HIF-2α binds to other factors and undergoes conformational changes in the nucleus, thereby reducing its immunoreactivity [30]. Recently, Nilsson et al [31] reported that the immunohistochemical expression of HIF-2α was detectable in most neuroblastomas, whereas HIF-1α protein was primarily restricted to cells surrounding necrotic areas. These observations suggest that expression of the HIF-2α pathway may also be associated with dysregulated oncogenic pathways regardless of the presence of hypoxic conditions.

Because the inactivation of VHL results in increased cellular HIF-1α and HIF-2α expressions [32,33], we also examined the expression of VHL in ovarian carcinomas. Immunohistochemical analysis showed a tendency toward a decreased expression of VHL in carcinomas as compared with benign tumors. LOH at the VHL locus was detected in 24% of ovarian carcinomas but did not show a significant correlation with loss of VHL expression. Microsatellite markers used in this study are known to be closely associated with the *VHL* gene and have previously been used as VHL markers [21,22]. However, they are not within the *VHL* gene, and this might have contributed in part to the dissociation between LOH and expression of VHL. Interestingly, the expressions of VHL and HIF-1α were inversely correlated based on the statistical analysis and topological distribution in clear cell carcinomas. These findings postulate that the decreased expression of VHL may have a role in the development of clear cell carcinomas of the ovary via upregulating the expression of HIF-1α.

The activation of HIF in cancer has been shown to contribute to tumor angiogenesis. We previously reported

that ovarian carcinoma cells at the tip of the papillary projection apart from blood vessels exhibit stronger expression of VEGF [34]. In this study, therefore, we examined whether VEGF and MVD as a marker of angiogenesis are associated with HIF-1α or HIF-2α expression. We found a positive correlation between nuclear HIF-1α and VEGF but not with MVD. In endometrial carcinoma, HIF-1α was significantly correlated with tumor MVD, whereas in lung carcinoma, only HIF-2α expression was significantly correlated with tumor MVD [23,24]. Accordingly, the relative importance of HIF-1α and that of HIF-2α in tumor angiogenesis may differ among cancer types. In ovarian carcinomas, although VEGF overexpression has been reported on [35-37], there has been controversy about the correlation of angiogenesis presented as MVD with the expression of VEGF and patient survival [37]. Further studies on other angiogenetic factors are needed to clarify the key molecule [15] and the association between the HIF system and vascularization in ovarian carcinoma.

These in vivo findings strongly suggest that nuclear HIF-1α has prognostic importance in ovarian carcinomas. On the other hand, upregulation of HIF-2α may also play an important role in oncogenesis and the progression of ovarian carcinoma. Over the last several years, HIF-1 has emerged as an attractive target for cancer therapy [7,38]. These results support the hypothesis that the HIF system could be an important molecular target in the treatment of ovarian carcinoma. In addition, it may be possible to identify subgroups of patients with ovarian carcinoma who are potential candidates for clinical trials aimed at inhibiting the HIF pathway.

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