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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 microenvironmental 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 microvessel 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 microdissected 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 ($P =$

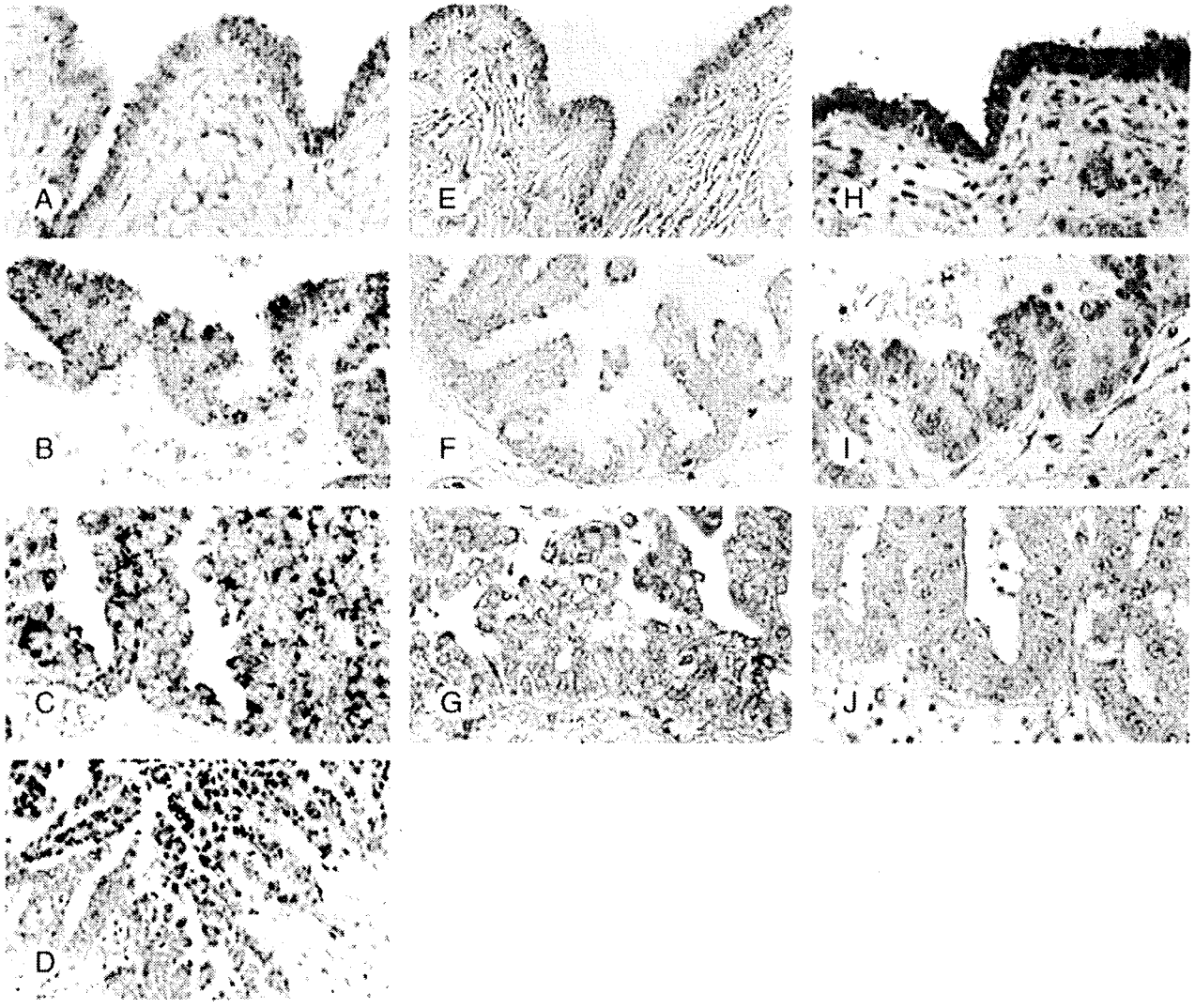

Fig. 1 Immunohistochemical staining of HIF-1α (A-D), HIF-2α (E-G), and VHL (H-J) in various epithelial ovarian tumors. A, Serous cystadenoma. B, Serous borderline tumor. C, Serous adenocarcinoma. D, Serous adenocarcinoma for HIF-1α. E, Serous cystadenoma. F, Serous borderline tumor. G, Serous adenocarcinoma 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

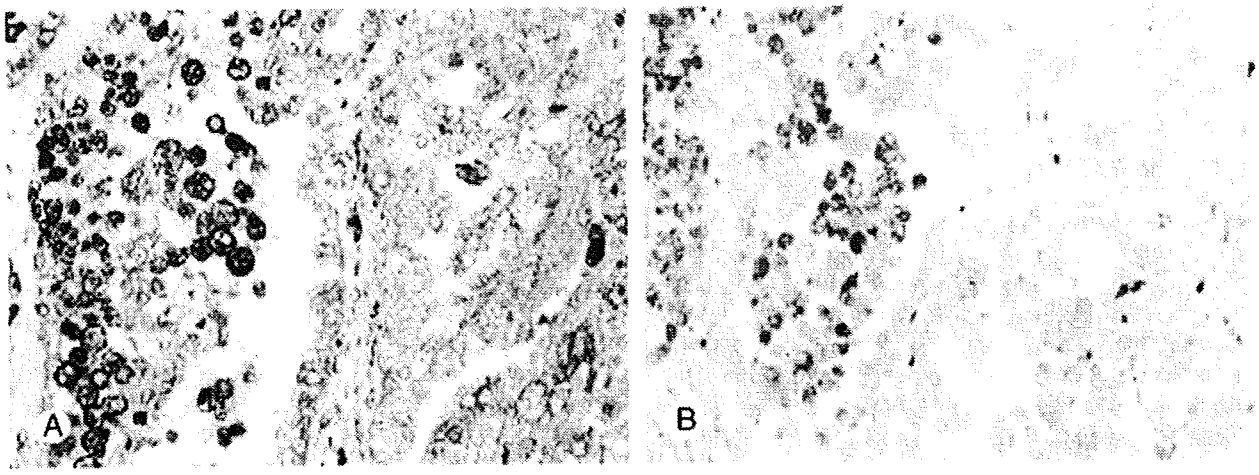

Fig. 2 Serial sections for the immunolocalizations of HIF-2α (A) and CD68 (B) in serous adenocarcinomas. A, Positive expression of HIF-2α in tumor cells and macrophages (original magnification ×250). B, Serial section showing CD68-positive macrophages (original magnification ×250).

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

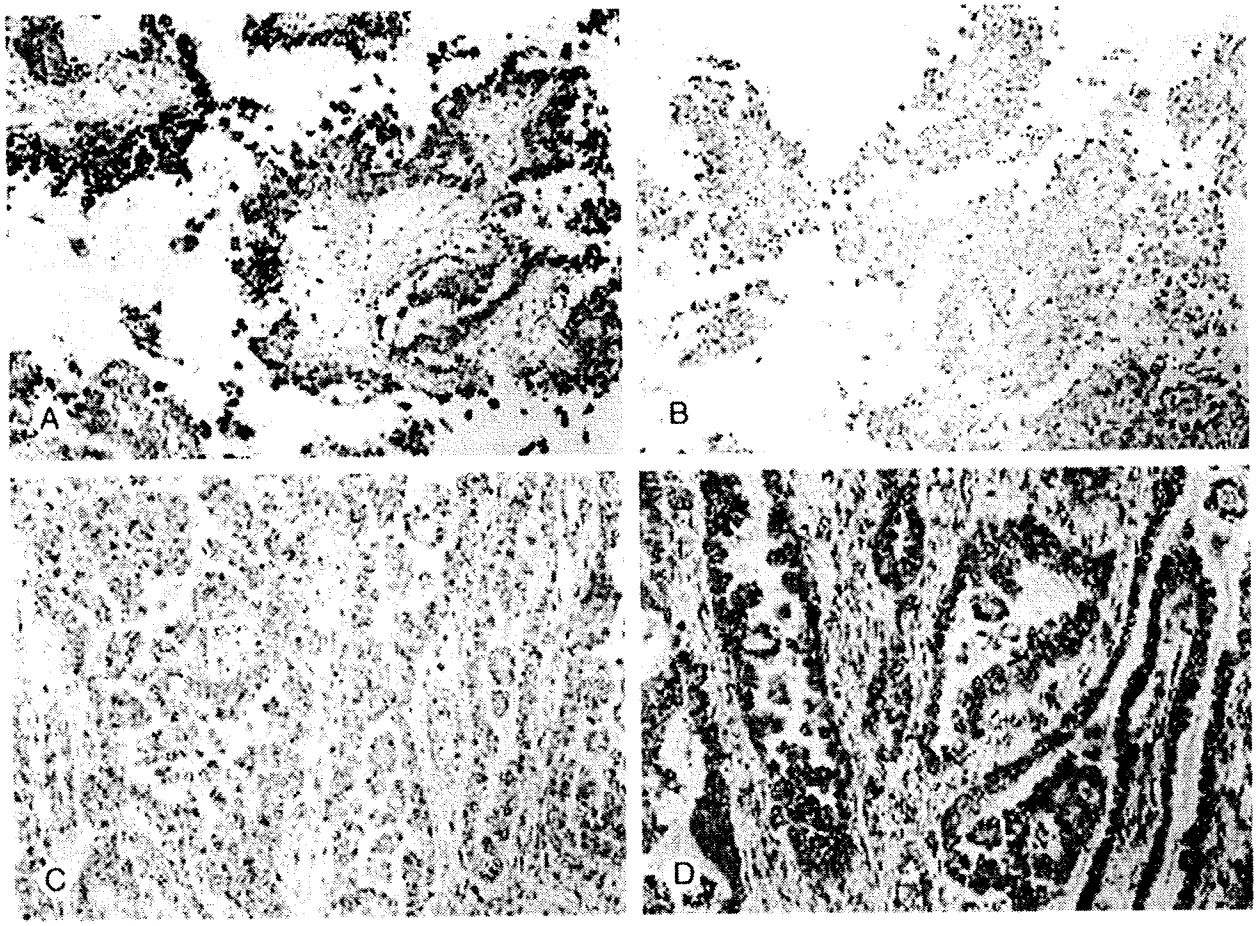

Fig. 3 Topological correlation between HIF-1α and VHL. In serial sections for the immunolocalizations of HIF-1α and VHL, the tumor cells with cytoplasmic expression of HIF-1α (A and C) are associated with reduced or loss of VHL expression (B and D), compared with the surrounding tumor cells negative for HIF-1α (original magnification ×100).

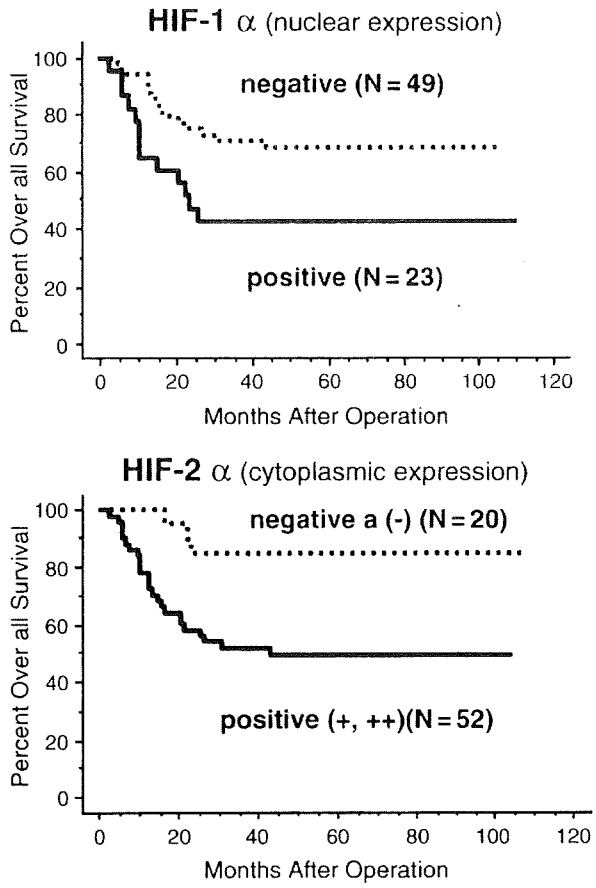

Fig. 4 Overall survival of patients with ovarian carcinoma according to the expressions of HIF-1α (A) and HIF-2α (B). Kaplan-Meier analysis showed that the prognosis was significantly poorer in patients with positive nuclear immunostaining for HIF-1α. Patients with positive HIF-2α showed significantly 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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Hypoxia inducible factor 1-α expression as a factor predictive of efficacy of taxane/platinum chemotherapy in advanced primary epithelial ovarian cancer

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Abstract

To investigate the impact on survival of HIF 1-α expression on primary advanced epithelial ovarian cancer (EOC), we examined the correlations between prognosis and HIF 1-α expression by Western blot analysis in 52 cases of stage III/IV EOC. HIF 1-α expression was confirmed in 36 cases (69.2%) of EOC, and HIF 1-α-expressing tumors had a significantly higher rate of response ($p < 0.01$) to postoperative paclitaxel/carboplatin combination chemotherapy (TC) than tumors without HIF1-α expression. Moreover, patients with HIF 1-α-expressing tumors with suboptimal resection of stage III/IV tumors indicated for postoperative TC exhibited significantly better survival ($p < 0.01$).

Keywords: HIF 1-α; Epithelial ovarian cancer; Chemotherapy; Prognostic factor

1. Introduction

Hypoxia inducible factor 1-α (HIF 1-α) has been reported to be an important predictor of tumor progression for several types of solid cancers [1–5]. However, although several *in vitro* studies have reported correlations between HIF 1-α expression and cell biological features in ovarian cancer, study of the clinical significance of HIF 1-α still has been limited [6]. To determine the clinical usefulness of HIF 1-α expression in treatment of primary epithelial ovarian cancer (EOC), we examined whether HIF 1-α expression can predict effects of postoperative induction chemotherapy and long-term prognosis in patients with stage III/IV advanced EOC.

2. Materials and methods

The study included 52 cases of stage III/IV EOC. Fourteen patients underwent optimal resection (residual tumor <1 cm), while 38 patients underwent suboptimal resection at primary surgery. Furthermore, all patients with suboptimal resection had measurable disease usable for determining direct effects of TC. The clinicopathological characteristics of patients did not differ significantly between optimal resection and suboptimal resection as summarized in Table 1. All of the patients were indicated for postoperative TC (175–180 mg/m^2 paclitaxel and a dose of carboplatin an area under the concentration curve

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Table 1
Correlations between HIF-1 α expression and clinicopathologic factors

Factors	HIF-1 α[a] positive	HIF-1 α negative
Total number of cases	36	16
Mean ages (range)	57.9 ± 8.2 years (34–84)	57.2 ± 7.3 years (39–73)
FIGO stage (%)[b]		
Stage III	31 (68.9)	14 (31.1)
Stage IV	5 (71.4)	2 (28.6)
Histologic subtype (%)		
Serous	19 (65.5)	10 (34.5)
Endometrioid	7 (77.8)	2 (22.2)
Mucinous	3 (60.0)	2 (40.0)
Clear-cell	7 (77.8)	2 (22.2)
Histologic grade (%)[c]		
Grade 1	13 (68.4)	6 (31.6)
Grade 2	10 (71.4)	4 (28.6)
Grade 3	6 (60.0)	4 (40.0)
Surgical status (%)		
Optimal surgery	9 (64.3)	5 (35.7)
Sub optimal surgery	27 (77.1)	11 (28.9)
Overall response rate of postoperative chemotherapy (%)	18 (66.7)	5 (45.5)**
Complete response rate of postoperative chemotherapy (%)	13 (48.1)	2 (18.2)**

[a] HIF, hypoxia inducible factor.
[b] FIGO, Federation of International Gynecology and Obstetrics.
[c] Not including clear-cell carcinomas.
** $p < 0.01$.

by Calvert's formula of 5–6). Direct effects of chemotherapy were assessed using the World Health Organization criteria. HIF 1-α expression was determined by Western blot analysis using anti-HIF 1-α (Novus Biologicals, Littleton, CO) for stocked fresh-frozen tissues, and if an independent positive band in the region of 120 kDa was confirmed on quantification using NIH image analysis, it was taken to indicate HIF 1-α expression (Fig. 1). We obtained fully informed written consent from all patients prior to obtaining the specimens. We used the chi-square test and log-rank test for statistical analysis, with p-values less than 0.05 considered significant.

3. Results

HIF 1-α expression was confirmed in 36 (69.2%) of the patients with FIGO stage III/IV tumors, and no significant correlation was observed between frequency of HIF 1-α expression and patient age, histologic subtype, histologic grade, FIGO stage (III or IV), or surgical status (optimal or suboptimal resection). However, HIF 1-α-expressing tumors exhibited significantly higher overall response rate ($p < 0.01$) and complete response rate ($p < 0.01$) to TC than tumors without HIF 1-α expression (Table 1). Moreover, HIF 1-α predicted prognosis for neither the group of all stage III/IV patients nor that with optimal resection. Although no significant differences were noted in clinicopathologic characteristics between patients with optimal and those with suboptimal resection (Table 2), but among patients in stage III/IV who underwent suboptimal resection at primary surgery and were indicated for postoperative TC, those with HIF 1-α-expressing tumors had a significantly better prognosis than those with tumors without HIF 1-α expression (Fig. 2).

4. Discussion

HIF 1-α expression in malignant tumors has been reported as a predictive factor for tumor progression and a prognostic factor correlated with angiogenesis. However, HIF 1-α expression in solid cancers exhibits marked variation among primary organs in the English literature [1–5]. Generally, HIF 1-α predicts tumor progression, and HIF 1-α-

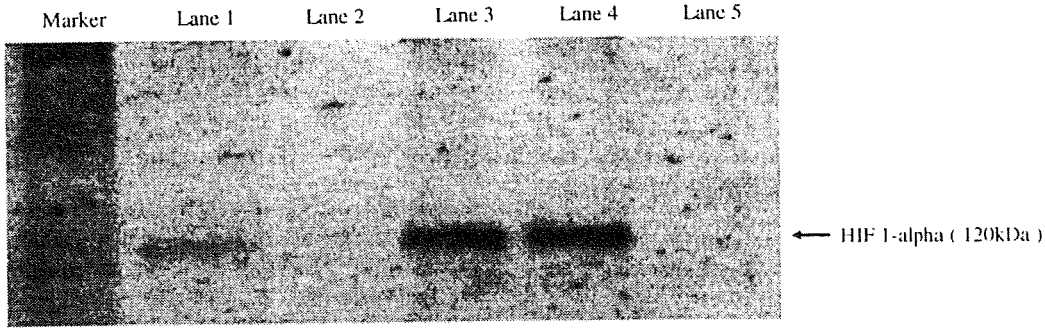

Fig. 1. The expression of HIF 1-α proteins detected by Western blotting. Lane 1: positive control; HCT-116 cell were grown in a chamber containing 1% oxygen, 5% carbon dioxide, and 94% nitrogen at 37 degree for 3 days. Lane 2: negative control; without primary antibody. Lane 3 and 4: HIF 1-α positive cases. Lane 5: HIF 1-α negative case.

Table 2
Clinicopathologic characteristics of all patients

Factors	Optimal	Suboptimal
Total number of cases	14	38
Mean ages (range)	59.6 + 8.3 years (46–84)	57.1 ± 7.6 years (34–74)
FIGO stage (%)[a]		
Stage III	13 (92.8)	32 (84.2)
Stage IV	1 (7.2)	6 (15.8)
Histologic subtype (%)		
Serous	9 (64.3)	20 (52.6)
Endometrioid	2 (14.3)	7 (18.4)
Mucinous	1 (7.1)	4 (10.6)
Clear-cell	2 (14.3)	7 (18.4)
Histologic grade (%)[b]		
Grade 1	6 (50.0)	13 (41.9)
Grade 2	4 (33.3)	10 (32.3)
Grade 3	2 (16.7)	8 (25.8)
Mean treatment courses (range)	5.9 ± 0.3 course (4–6)	5.8 ± 0.9 courses (3–6)
Mean follow up period (range)	58.4 ± 31.4 months (13–135)	48.3 ± 26.3 months (8–110)

[a] FIGO, Federation of International Gynecology and Obstetrics.
[b] Not including clear-cell carcinomas.

expressing cancers tend to have a poor prognosis. However, Nakayama et al. [6] reported finding no relationship between HIF 1-α expression and intratumoral microvessel density, and that vascular endothelial cell growth factor (VEGF) up-regulated HIF 1-α gene, though levels of expression of neither gene affected the survival of patients with EOC. Furthermore, Birner et al. [7] examined HIF 1-α expression in 102 cases of FIGO stage I–IV EOC by immunohistochemical staining, reported that 68.6% of cases of EOC expressed HIF 1-α, and concluded that HIF 1-α protein overexpression also has no impact on prognosis and that response to TC is independent of HIF 1-α expression. However, Escuin et al. [8] recently found that microtubule-targeting drugs, such as taxanes, could be effective in down-regulating HIF 1-α protein via effects on microtubule cytoskeleton that are correlated with HIF 1-α translation activity. For patients with suboptimally resected advanced EOC, survival impact is closely related to effects of postoperative chemotherapy. Therefore, because paclitaxel may exhibit anti-angiogenetic effects through down-regulation of HIF 1-α protein expression, the survival impact of HIF 1-α expression on EOC may be noted only in patients who are stage III/IV, have undergone

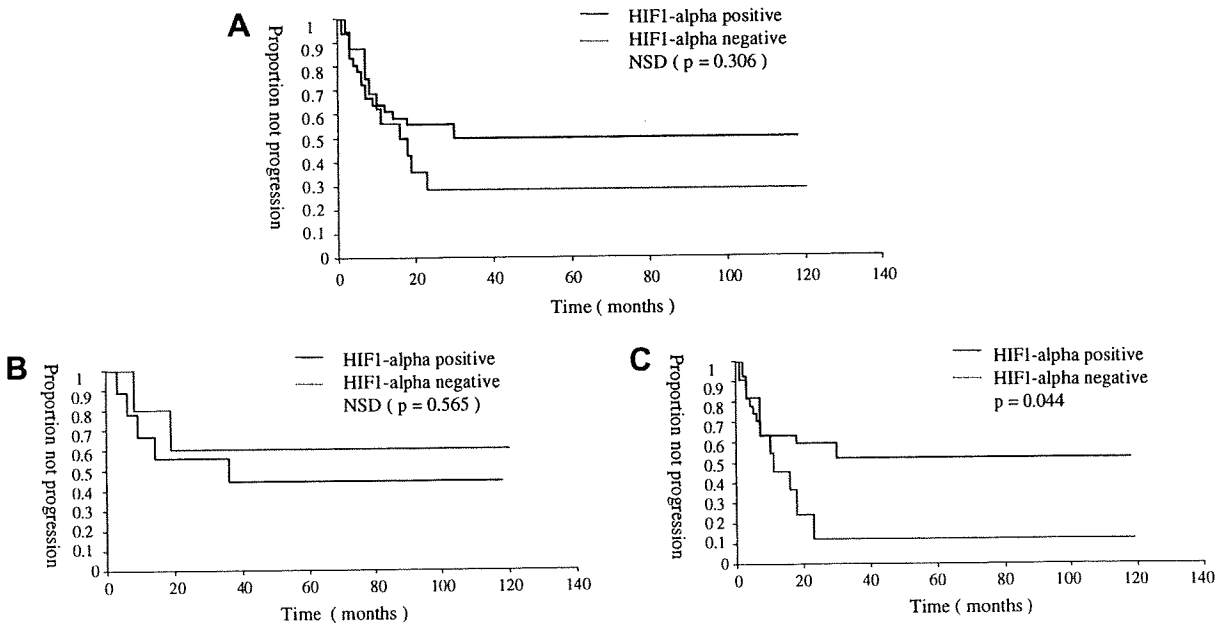

Fig. 2. Correlation between survival and HIF 1-α expression in stage III/IV epithelial ovarian cancer. (A) Progression-free survival in the group of all stage III/IV patients. (B) Progression-free survival of stage III/IV patients who underwent optimal resection at primary surgery and were indicated for postoperative paclitaxel/carboplatin chemotherapy. (C) Progression-free survival of stage III/IV patients who underwent suboptimal resection at primary surgery and were indicated for postoperative paclitaxel/carboplatin chemotherapy. *p*-values were calculated with the log-rank test.

suboptimal resection at primary surgery, and are indicated for postoperative TC. Although TC has been widely used as an effective standard regimen of chemotherapy for primary or recurrent EOC, and TC has achieved a 65–75% overall response rate in several phase 3 clinical trials [9,10], no factors predictive of TC have been found. The present findings suggest that although expression of HIF 1-α is not a factor predictive of survival of patients with early-stage or optimally resected advanced EOC, it does predict the efficacy of chemotherapy using TC. Furthermore, determination of HIF 1-α expression should be useful for devising individualized treatment regimens for advanced EOC. Clinical trials targeting HIF 1-α treatment using taxanes are needed to improve the long-term prognosis of patients with suboptimally resected advanced EOC.

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ORIGINAL REPORT

PIEPOC: A New Prognostic Index for Advanced Epithelial Ovarian Cancer—Japan Multinational Trial Organization OC01-01

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A B S T R A C T

Purpose
The purpose of this study was to construct a simple and powerful prognostic index (PI) of epithelial ovarian cancer, the PIEPOC.

Patients and Methods
In a retrospective review, data from 768 women with stage III or IV epithelial ovarian cancer from 24 institutions in Japan were evaluated for clinical features predictive of overall survival. A PI and risk groups to predict overall survival after initial surgery were developed using the proportional hazards regression model.

Results
Of six factors, the four prognostic factors that remained independently significant in the analysis of a training sample (538 randomly selected patients) were age, performance status (PS), histologic cell type, and residual tumor size. From the regression function, we derived a PI = 1 (if age 70 and above) + 1 (if PS 1 or 2) + 2 (if PS 3 or 4) + 1 (if mucinous or clear-cell) + 2 (if residual size 0.1 cm and above). Patients were classified into three risk groups (PIEPOC): low risk (PI 0-2), intermediate risk (PI 3), and high risk (PI 4-6). The PIEPOC was equally predictive in a validation sample (n = 230), identifying three groups (5-year survival: 0.67 in low, 0.43 in intermediate, 0.17 in high risk).

Conclusion
Our proposed PI, the PIEPOC, was predictive in our patient population and may have utility in clinical practice. Prospective studies would be needed to confirm the prognostic predictive ability of the PIEPOC for patients with advanced epithelial ovarian cancer.

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INTRODUCTION

Ovarian cancer is the leading cause of death among female cancer patients worldwide.[1] Although mortality from ovarian cancer in Japan is relatively low compared with other developed countries, the mortality and incidence of ovarian cancer in the Japanese population have been increasing since the 1970s.[2]

In patients with advanced epithelial ovarian cancer, several studies have identified age, performance status (PS), histologic cell type, stage, histologic grade, residual tumor size, and presence of ascites as independent prognostic factors.[3-6] A Dutch study group identified PS, residual tumor size, stage, histologic grade, and ascites as prognostic factors using data from two clinical trials.[3] On the basis of these prognostic factors, Lund et al compared the prognostic index (PI) of Dutch study and a Danish PI including PS, residual tumor size, age, and weight or body surface area from a clinical trial and proposed a final PI including information on PS and residual tumor size.[7] Those PIs for survival were developed for planning of treatment for individual patients and stratifying patients in further clinical trials.[3,7] Although they proposed a simple two-covariate PI after validating statistical models in two well-defined independent patient populations, the classification method of risk groups according to the PI was not well specified.[7] The identification of different risk groups should have important therapeutic implications. The purpose of this study was to develop a better prognostic-factor model and to construct a simple and powerful PI of epithelial ovarian cancer by using data from a long-term follow-up study.

PATIENTS AND METHODS

Participants

The study participants were patients with FIGO (International Federation of Gynecology and Obstetrics) stage III or IV epithelial ovarian cancer who were treated with adjuvant chemotherapy after maximal surgical debulking between 1994 and 2000 at 24 institutions in Japan (Japan Multinational Trial Organization OC01-01).[8] In the consecutive series of 880 women, information regarding important patient characteristics was not available for 112 patients (68 for PS, 16 for histologic cell type, and 30 for residual tumor size). Thus, data from 768 women were included in the present study and evaluated for clinical features predictive of overall survival. The patient characteristics evaluated for potential prognostic importance were age, Eastern Cooperative Oncology Group PS, FIGO stage, histologic cell type, histologic grade, and residual tumor size. The presence of ascites was not assessed because the study subjects were patients with surgically confirmed stage III or IV ovarian cancer and we gave greater importance to the surgical findings than to the ascites itself. Overall survival was defined as time from the initial surgery until death resulting from any cause.

Statistical Analysis

A data set was randomly split into training sample for model development and validation sample for model validation for evaluating reproducibility of prognostic-factor model. The survival curves were estimated with the Kaplan-Meier method. The univariate association between potential prognostic factors and overall survival were analyzed with the log-rank test. A PI to predict overall survival was developed using proportional hazards regression model with backward elimination methods. Additivity assumption of the model was verified by the pooled interaction test. We selected the best risk classification in an attempt to separate the prognosis of patients based on the Akaike's information criterion (AIC).[9]

The model performance was assessed with respect to calibration and discrimination. Calibration was examined with graphical expressions (calibration curves) of the relationship between the observed 5-year Kaplan-Meier estimates of overall survival and the predicted probabilities for each group. We used bootstrapping with 200 repetitions to obtain relatively unbiased estimates. Discrimination was evaluated with the concordance index (c index), which is the proportion of all pairs of patients whose survival time can be ordered such that the patient with the lower risk is the one who survived longer.[10] Statistical analyses were done by using SAS version 9.1 (SAS Institute, Cary, NC) and *S*-Plus version 6J (Mathematical Systems Inc, Tokyo, Japan) with the Design and Hmisc libraries added.

Table 1. Characteristics of 768 Patients and Outcome According to Patient Characteristics

Characteristic	No.	%	Overall Survival 5-Year Survival (%)	*P*
Age, years				.007
≤ 39	50	7	54	
40-49	181	24	47	
50-59	257	33	45	
60-69	191	25	49	
≥ 70	89	12	31	
Performance status				< .001
0	308	40	61	
1	293	38	39	
2	102	13	34	
3	43	6	21	
4	22	3	17	
FIGO stage				< .001
IIIA	22	3	79	
IIIB	68	9	59	
IIIC	524	68	46	
IV	154	20	31	
Histologic cell type				.022
Serous	505	66	45	
Mucinous	56	7	43	
Endometrioid	101	13	51	
Clear cell	51	7	36	
Mixed epithelial	14	2	50	
Others	41	5	36	
Histologic grade				.144
1	121	24	51	
2	146	29	35	
3	235	47	44	
Unknown	266			
Residual tumor size, cm				< .001
0 (microscopic)	119	16	70	
0.1-0.9	129	17	54	
1.0-1.9	71	9	51	
≥ 2.0	449	58	35	

RESULTS

Of 768 patients, 408 patients had died, and the median follow-up times for all patients or 360 surviving patients were 4.1 year or 4.2 years, respectively. The patient characteristics and the 5-year survival probability according to the factors are shown in Table 1. All characteristics except for histologic grade were significantly related to overall survival by the univariate analysis.

We randomly selected 538 patients (70% of all patients) as a training sample in which to identify independent prognostic factors for building a model. Prognostic factors that remained independently significant in the multivariate analysis of the training sample were age, PS, cell type, and size of residual disease. After combining levels of factors that appeared to have a similar effect on survival and checking additivity of effects by pooled interaction tests ($P = .667$), the characteristics and categories that remained independently significant were age (≤ 69 *v* ≥ 70 years), PS (0 or *v* 1 or 2 or *v* 3 or 4), cell type (mucinous or clear-cell *v* others), and residual tumor size (0 *v* ≥ 0.1 cm; Table 2). A linear function based on estimated regression coefficients was as follows: 0.448 (if age 70 years and older) + 0.539 (if PS 1 or 2) + 0.980 (if PS 3 or 4) + 0.488 (if mucinous or clear-cell) + 0.943 (if residual size 0.1 cm and above). From the weight of variables in the

Table 2. Final Prognostic-Factor Model in the Training Sample (n = 538)

Factors	Hazard Ratio	95% CI	*P*
Age, years			
≤ 69	1.00	—	—
≥ 70	1.57	1.11 to 2.20	.010
Performance status			
0	1.00	—	—
1 or 2	1.71	1.31 to 2.24	< .001
3 or 4	2.67	1.79 to 3.96	< .001
Histologic cell type			
Others	1.00	—	—
Mucinous or clear cell	1.63	1.14 to 2.33	.007
Residual tumor size, cm			
0 (microscopic)	1.00	—	—
≥ 0.1	2.57	1.67 to 3.95	< .001

function, we derived a simplified PI as follows: PI = 1 (if age 70 and above) + 1 (if PS 1 or 2) + 2 (if PS 3 or 4) + 1 (if mucinous or clear-cell) + 2 (if residual size 0.1 cm and above).

The PI of patients in the training sample was distributed between 0 and 5 (0, n = 46; 1, n = 31; 2, n = 143; 3, n = 223; 4, n = 88; 5, n = 7). We selected best classification among all possible classification in an attempt to separate the prognosis of patients with respect to the AIC. The total number of examined classification was 15, including five for two categories and 10 for three categories. As a result, patients were classified into three risk groups, named PIEPOC (PI of Epithelial Ovarian Cancer): low-risk group (PI 0 to 2), intermediate-risk group (PI 3), and high-risk group (PI 4 to 6). The PIEPOC was equally predictive in a randomly selected validation sample (n = 230), identifying three groups (5-year survival probability: 0.67 in low-risk group, 0.43 in intermediate-risk group, 0.17 in high-risk group; Fig 1). If a reference category was the low-risk group, the hazard ratio was 2.29 (95% CI, 1.44 to 3.65) in the intermediate-risk group and 4.87 (95% CI, 2.97 to 7.98) in the high-risk group. This predictability was reproducible in all patients (Fig 2A) and stage IIIc or IV patients (Fig 2B).

The PIEPOC was well calibrated to predict 5-year survival in the all patients, although overestimation (3.0% in the low-risk group) and underestimation (0.8% in the intermediate-risk group and 1.3% in the high-risk group) were observed (Fig 3). The calibration curve was similar to that both in the training sample and the validation sample. The estimated c index in the training sample, the validation sample, and all patients were 0.63, 0.67, and 0.64, respectively. The c index for the PI (0 to 6; seven groups) was 0.65 in all patient; thus, the difference of c index between the PI and the PIEPOC (three groups) was only 0.01.

DISCUSSION

We developed a PI to differentiate risk groups among advanced epithelial ovarian cancer on the basis of demographic, clinical and pathologic characteristics of patients. Accuracy of the simple risk group model was statistically evaluated with respect to discrimination and calibration and reproducibility of the model was accessed by data-splitting method.[10] Analyses for prognostic factors in advanced epithelial ovarian cancer have been carried out since the late 1980s. The Gynecologic Oncology Group in the United States performed a prognostic factor analysis using data from six clinical trials (n = 2,123) and identified age, PS, and residual tumor size as independent significant factors for predicting survival.[5] A Dutch study group identified PS, residual tumor size, FIGO stage, histologic grade by Broders' classification, and ascites as prognostic factors using data from two clinical trials (n = 268).[3] On the basis of the analysis by the Dutch study group, Lund et al compared the PI of Dutch study and a Danish PI including PS, residual tumor size, age, and weight or body-surface area from a clinical trial (n = 301) and proposed a final simple PI including

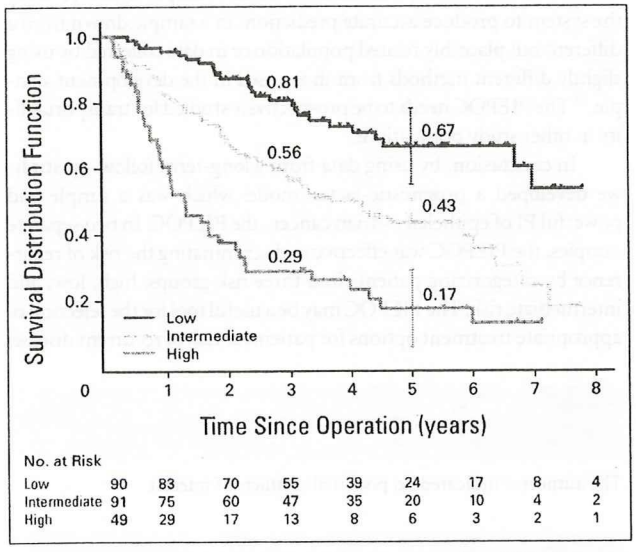

Fig 1. Survival curves according to risk group based on PIEPOC, a new prognostic index of epithelial ovarian cancer, in the validation sample. Bars indicate 95% CIs.

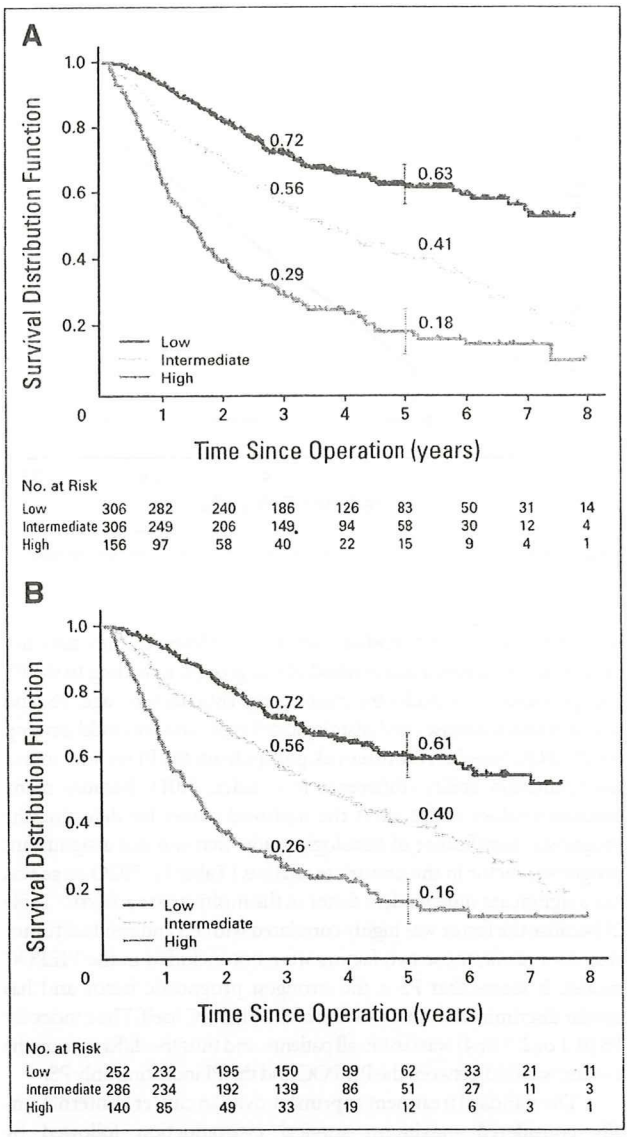

Fig 2. Survival curves according to risk group in (A) all patients and (B) stage IIIC or IV patients. Bars indicate 95% CIs.

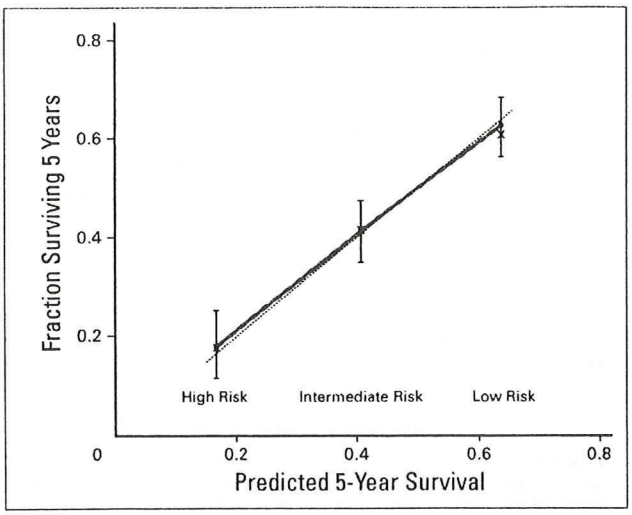

Fig 3. Calibration curve for 5-year survival in all patients. x, bias-corrected calibration.

information on PS and residual tumor size.[7] However, they have not proposed the classification method of risk groups according to the PI. Our proposed PI includes the major prognostic factors (age, PS, and size of residual disease) and histologic cell type, and we could develop the PIEPOC based on the three risk groups from the PI without loss of discrimination ability (difference in c index, 0.01). Because many unknown values might affect the statistical power for detecting the prognostic significance of histologic grade, that was not a significant prognostic factor in the univariate analysis (Table 1). FIGO stage was not a significant independent factor in the multivariate analysis (Table 2) because the factor was highly correlated with PS and residual tumor size. As a result, those two factors were not included in the PIEPOC model. It seems that PS is the strongest prognostic factor and has similar discrimination ability to that of the PIEPOC itself. The c index for PS (0, 1 or 2, 3 or 4) was 0.61 in all patients, and thus the difference on the c index was 0.03 between the PIEPOC and the PI including only PS.

The standard treatment of primary ovarian cancer is internationally considered maximum surgical cytoreduction followed by platinum-based chemotherapy.[11] In our cohort, the patients were treated with paclitaxel + cisplatin/carboplatin (30%), cyclophosphamide + doxorubicin + cisplatin (26%), cyclophosphamide + cisplatin/carboplatin (11%), cisplatin + carboplatin (4%), cisplatin + irinotecan (2%), docetaxel + carboplatin (2%), or other regimens including single agent or other combinations (25%). Although a variety of treatment regimens have been used in the study period and the heterogeneity of treatments is a limitation of this type of study, most regimens may be considered standard chemotherapy in advanced ovarian cancer during the study period. Additionally, the 5-year survival probabilities in patients with the platinum-based regimens (n = 332) were 0.60 in the low-risk group, 0.41 in the intermediate-risk group, 0.22 in the high-risk group. If a reference was the low-risk group, the hazard ratios were 1.83 (95% CI, 1.32 to 2.54) in the intermediate-risk group and 4.38 (95% CI, 2.92 to 6.57) in the high-risk group. The 5-year survival probabilities in patients with the paclitaxel-platinum combination regimens (n = 229) were 0.79 in the low-risk group, 0.37 in the intermediate-risk group, 0.08 in the high-risk group. If a reference was the low-risk group, the hazard ratios were 3.27 (95% CI, 1.82 to 5.88) in the intermediate-risk group and 9.32 (95% CI, 5.04 to 17.2) in the high-risk group. As a result, the PIEPOC would also have predictive ability in the both treatment groups.

A meta-analysis reported that the median survival time ranged from 12 months to 62 months and that the mean weighted median survival time was 29 months among patients with stage III or IV ovarian carcinoma.[12] On the other hand, the survival time in our Japanese cohort was relatively longer than that in the Western population (median, 49 months; 95% CI, 40 to 55 months). One of the reasons there was a difference in survival time is that the year of the study period was relatively old in the meta-analysis (publication year 1989 to 1998) in comparison with the present study (operation year: 1994 to 2000). Thus, we may say that the Japanese population in our study is comparable to the Western population in terms of the similarity of administered treatments and long-term prognosis as well as identified prognostic factors.

The definitions of accuracy and generalizability with regard to assessment of a prognostic system have been discussed.[13] Accuracy (calibration and discrimination) is the degree to which predictions match observed outcomes. In the present study, although the errors in calibration were relatively small (0.8% to 3.0%; Fig 3) for 5-year survival probabilities, the discrimination based on c index was not very gratifying (0.64 in all patients). Although discrimination ability tends to be improved on more complex risk group models, we selected the simple risk group model because of making much account of generalizability. Generalizability (reproducibility and transportability) is the ability of a prognostic system to provide accurate predictions in a new sample of patients. Reproducibility requires the system to replicate its accuracy in patients who were not included in development of the system but who are from the same underlying population.[13] We evaluated the reproducibility by using data-splitting method because we had relatively large data sets. It might be reasonable to suppose that our classification is simple and reproducible without loss of discrimination ability because the best c index for a PI based on a six-covariate full model was 0.68 in all patients and the gain of discrimination ability was relatively small. Transportability requires the system to produce accurate predictions in a sample drawn from a different but plausibly related population or in data collected by using slightly different methods from those used in the development sample.[13] The PIEPOC needs to be prospectively studied for transportability in other study populations.

In conclusion, by using data from a long-term follow-up study, we developed a prognostic-factor model which was a simple and powerful PI of epithelial ovarian cancer, the PIEPOC. In two separate samples, the PIEPOC was effective in discriminating the risk of recurrence by categorizing patients into three risk groups: high, low, and intermediate risk. The PIEPOC may be a useful tool for the selection of appropriate treatment options for patients at risk of recurrent disease.

The author(s) indicated no potential conflicts of interest.

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