EJGO European Journal of Gynaecological Oncology

Differential regulation and prognostic significance of endogenous hypoxic markers in endometrial carcinomas

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Summary

Tumor hypoxia is associated with malignant progression and treatment resistance. Hypoxia-related factors (HRFs), such as hypoxia-inducible factor 1α (HIF-1α), carbonic anhydrase IX (CA IX), and glucose transporter-1 (GLUT-1) permit tumor cell adaptation to hypoxia. The authors attempted to elucidate the correlation of these markers with variable clinicopathological factors and overall prognosis. Immunohistochemistry for HIF-1α, CA IX, and GLUT-1 was performed on formalin-fixed, paraffin-embedded tissues from 140 cases of endometrial carcinomas. Expression of HIF-1α, CA IX, and GLUT-1 was shown in 33.6% (47 cases), 47.9% (67 cases), and in 67.1% (94 cases) HIF-1α and GLUT-1 expression was significantly associated with myometrial invasion. CA IX expression was associated with histologic grade and tumor stage, as well as myometrial invasion. HIF-1α expression was associated with a shorter OS. The present study suggests that expression of these hypoxia-related factors tends to be associated with poor prognosis, and HIF-1α expression is an independent unfavorable prognostic factor in endometrial carcinomas.

Key words: Endometrial carcinoma; Hypoxia; HIF-1α; CA IX; GLUT-1.

Introduction

Endometrial carcinoma (EC) is one of the most common malignancies that occur in the female reproductive system. In the US, it is estimated that approximately 49,560 new cases of EC with 8,190 deaths will occur in 2013 [1]. In Korea, its incidence has been dramatically increased, thus accounting for approximately 16% of total gynecologic malignancies [2]. There are two types of EC depending on the clinicopathologic characteristics [3, 4]. Type I ECs are characterized by the endometrioid histology, accounting for approximately 80% of total ECs. It is known that type I ECs are hormone dependent, show a predilection in younger patients, and are associated with a good prognosis. In addition, they are also characterized by a high incidence of loss-of-function alterations in the PTEN tumor suppressor gene, as well as defects in DNA mismatch repair genes. By contrast, type II ECs usually have non-endometrioid histology, are not estrogen-dependent, are seen in older patients, and are associated with a poor prognosis. In addition, they are likely to harbor p53 mutation. The prognosis of EC is dependent on several factors, including the stage, histologic grade, histopathologic subtype, and invasion of myometrium [5].

In carcinogenesis, hypoxia renders a more aggressive phenotype, with increased invasiveness, proliferation and metastasis, and poorer survival rate. Additionally, several clinical studies have demonstrated that hypoxia is associated with poor response to radiation and chemotherapy. Cellular adaptation to hypoxia represents a crucial step in tumor progression. Many solid tumors, including endometrial carcinomas, outgrow their own vasculatures beyond the size of several cubic millimeters, which results in hypoxia. In general, hypoxia within the tumor microenvironment is an important event in carcinogenesis because it can lead to the development of a more aggressive phenotype with increased invasiveness and proliferation, formation of metastasis, and poorer prognosis [6, 7]. Hypoxic malignant cells are more resistant to chemotherapy and radiotherapy [8]. For these reasons, hypoxia has been suggested to be an adverse prognostic factor for patient outcome. Cellular adaptation to hypoxia represents an essential step in tumor progression. All normal and neoplastic tissues are thought to possess a mechanism that helps survival in hypoxic conditions by modulating certain crucial genes. One of the key factors regulating cellular responses to hypoxia via transcription is hypoxia-inducible factor 1 (HIF-1). Thus, throughout the HIF-1-mediated pathway, various hypoxia-related factors (HRFs), such as carbonic anhydrase (CA) IX, glucose transporter-1 (GLUT-1), and vascular endothelial growth factor (VEGF) are activated [9-11]. HRFs

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Revised manuscript accepted for publication June 26, 2017

help cells to survive hypoxic stress by switching to anaerobic glycolysis and by increasing oxygen delivery (via angiogenesis) [12, 13].

Given the above background, the authors conducted this study to evaluate the prognostic value of hypoxia-related factors (HRFs), such as HIF-1α, CA IX, and GLUT-1 and to examine its correlation with the clinicopathologic parameters. To do this, they performed immunohistochemistry to determine the expression of these factors.

Materials and Methods

Formalin-fixed paraffin-embedded specimens were selected from 140 patients with EC who were diagnosed with EC and underwent surgical resection between January 1998 and December 2009 at Pusan National University Hospital, Busan, Korea.

Based on the primary pathology reports and the medical records of the patients, the authors collected the clinicopathologic data such as age, gender, tumor grading, histologic type, stage, lymphovascular invasion, and lymph node metastasis. Surgical staging was done based on the International Federation of Gynecology and Obstetrics (FIGO) criteria for EC; Stage I and II-IV were considered the early- and advanced-stage EC. Moreover, the histologic types and grades of EC were determined based on the World Health Organization (WHO) criteria. The overall survival (OS) was calculated from the date of surgery to that of death or the last follow-up visit. The current study was approved by the Institutional Review Board (IRB) at Pusan National University Hospital after obtaining informed consent.

Each slide was deparaffinized and rehydrated according to the standard procedure, and was treated with 0.01 mol/L sodium citrate buffer in a pressure cooker at 120°C for 15 minutes. Immunohistochemical staining was performed using the avidin-biotin peroxidase complex method with diaminobenzidine as a chromogen using the Vectastain ABC elite kit. Rabbit polyclonal antibodies CA IX (1:1000) and GLUT-1 (1:200), and mouse monoclonal antibodies HIF-1α (1:1000) were used as a primary antibody. Specimens of colon adenocarcinoma and renal cell carcinoma were used as positive controls for HIF-1α and CA IX, respectively, due to the known strong expression of these markers. Tumor capillaries were considered to be an internal positive control for GLUT-1.

Immunohistochemical staining was evaluated by two independent pathologists who were blinded to the specific diagnosis and prognosis for each individual case. Expression of HIF-1α was assessed by analyzing ≥ 1,000 tumor cells from tumor fields, and the labeling index was calculated as the percentage of labeled nuclei per total number of tumor cells that were counted. The immunoreactivity of HIF-1α was graded from 0 to 3+ (0, no staining; 1+, 1-25%; 2+, 26-50%; 3+, >50% nuclear staining) according to the nuclear expression, and only 3+ (>50% nuclear staining) was considered as a positive immunohistochemistry result [14, 15]. For GLUT-1 and CA IX, cases were considered positive if > 10% of their cells showed distinct membranous staining.[16,17]

A statistical analysis was conducted using SPSS 17.0 software. The associations between clinicopathological variables and the expression of HIF-1α, CA IX, and GLUT1 were assessed using Pearson's χ^2 test. OS was calculated using the Kaplan-Meier log-rank test. A multivariate analysis to assess their independent prognostic values was conducted using the Cox regression method. $P < 0.05$ was considered to indicate a statistically significant difference.

Table 1. — *Clinicopathological features of endometrial carcinoma patients (n=140).*

Parameters	n (%)
Histologic type	
Endometrioid	131 (93.6)
Non-endometrioid	9 (6.4)
Histologic grade	
1	49 (35.0)
2	62 (44.3)
3	30 (21.4)
Myometrial invasion	
< 1/2 of myometrium	92 (65.7)
≥ 1/2 of myometrium	48 (34.3)
FIGO Stage	
I	101 (72.1)
II	16 (11.4)
III	19 (13.6)
IV	4 (2.9)
LN metastasis	
Absent	122 (87.1)
Present	18 (12.9)
Overall survival	
Alive	114 (81.4)
DOD	26 (18.6)

DOD: died of disease.

Results

Clinicopathologic features are represented in Table 1. Based on the FIGO criteria, there were 101 cases in Stage I EC, 16 cased in Stage II EC, 19 cases in Stage III EC, and four cases in Stage IV EC. Histopathologic grading showed that there were 49 cases of G1, 61 cases of G2, and 30 cases of G3. In the present series, the median follow-up period was 125 (range 3 - 204) months. There were 114 (81.94) survivors at the last follow-up.

Expression of HIF-1α, CA IX, and GLUT-1 was observed in 33.6% (47/140 cases), 47.9 % (67/140 cases), and 67.1% (94/140 cases) of EC samples, respectively. HIF-1α expression was recognized through the nuclear staining of positive cells, whereas CA IX and GLUT-1 staining were distinct in the cell membrane. Representative cases of immunohistochemical staining of all markers are shown in Figure 1.

The correlations between clinicopathological variables and expression of HIF-1α, CA IX and GLUT-1 are shown in Table 2. Immunohistochemical analysis revealed a difference in the expression of HIF-1α, CA IX and GLUT-1 according to the depth of myometrial invasion. The rate of expression was significantly higher in ECs with deep myometrial invasion. The expression of CA IX was significantly associated with a higher histologic grade and

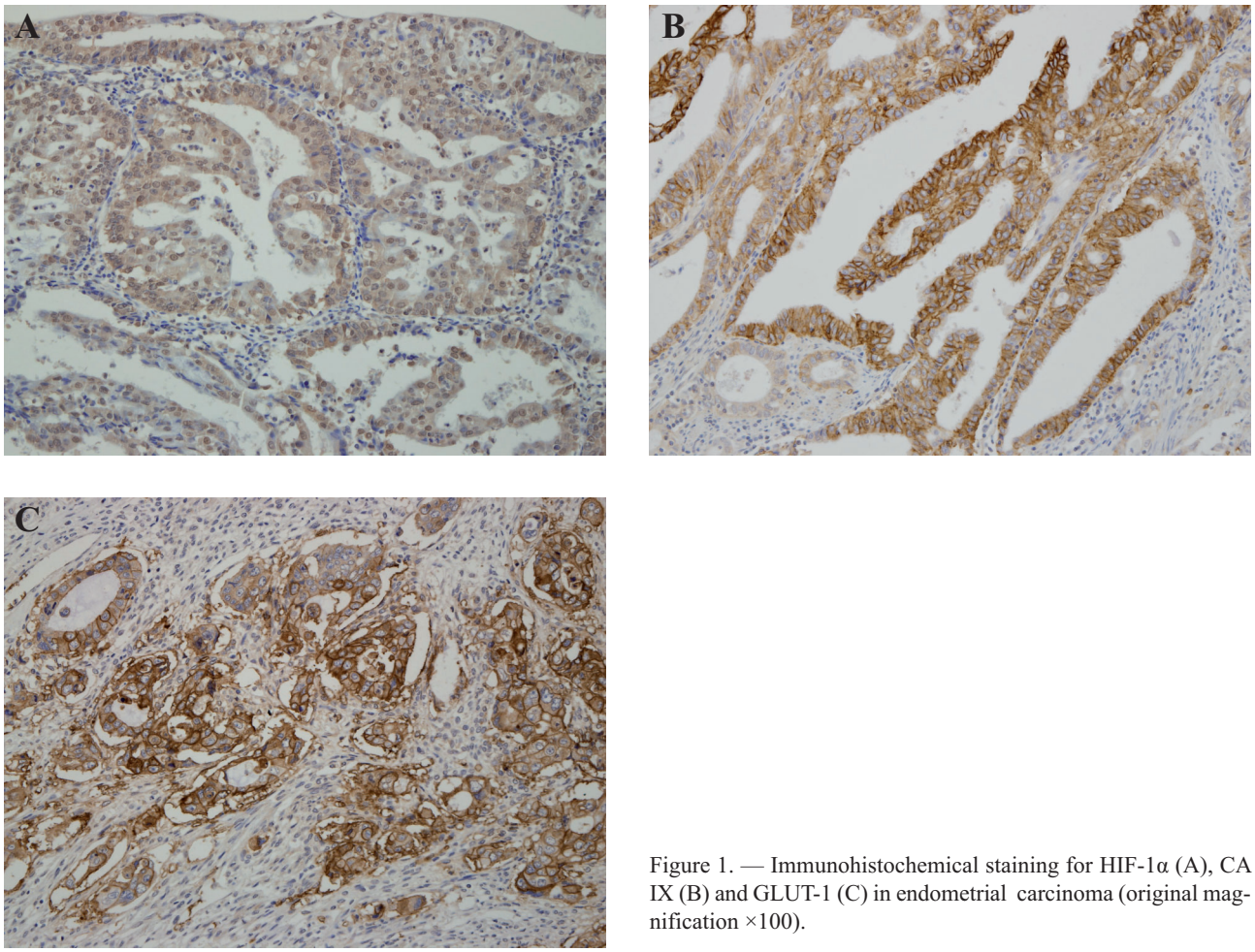

Figure 1. — Immunohistochemical staining for HIF-1α (A), CA IX (B) and GLUT-1 (C) in endometrial carcinoma (original magnification ×100).

advanced AJCC Stage in total cases of EC. The expression of HIF-1α was associated with poor survival rate.

The Kaplan-Meier survival analysis showed that the poor OS were associated with histologic grade, the FIGO Stage, myometrial invasion, lymph node metastasis, and expression of HIF-1α. The proportion of disease-related deaths was 36.2% (17/47) in the patients with HIF-1α expression and 9.7% (9/93) of those with no HIF-1α expression (p = 0.000) (Figure 2). On multivariate analysis of the variables defined in Table 3, there was a significant correlation between the expression of HIF-1α and shorter OS after the adjustment for histologic grade, the FIGO stage, lymph node metastasis, and myometrial invasion, all of which were significant variables on univariate analysis.

Discussion

Tumor hypoxia is known to affect patient prognosis as it leads to a more aggressive phenotype, with increased invasiveness, proliferation and metastases, resulting in a poorer survival rates [6, 7]. HIF-1α is essential for adapting the cellular environment to hypoxia by inducing the expression of various hypoxia response molecules, including CA IX and GLUT1. The evidence for these molecules as reliable markers of hypoxia has been reviewed elsewhere and the overexpression of HIF-1α, CA IX, and GLUT-1 in various malignant tumors has also been demonstrated [14-18].

HIF-1 has been known to be a key gene for adapting cells to microenvironmental conditions via the up-regulation of the transcription response to hypoxia [19]. Through the HIF-1-mediated pathway, various endogenous hypoxia markers used for the estimation of the oxygenation status of total ECs are activated; among them, CA IX and GLUT-1 have been known to play an important role [20, 21]. Previous studies have indicated that HIF-1α and its target genes act as positive regulators of tumor growth in many solid tumors, including ECs [18]. The present authors attempted to analyze the association between the prognosis and the expression of HRFs, including HIF-1α, CA IX, and GLUT-1 in cases of OECs.

Few reports have indicated that hypoxia and activation of HRFs may play a role in endometrial carcinogenesis.

Table 2. — *Association between HIF-1α, CA IX and GLUT-1 expression status and clinicopathological variables (n=140).*

Clinicopathologic parameters		HIF-1α			CA IX			GLUT-1	
	Positive (n=47)	Negative (n=93)	*p*	Positive (n=67)	Negative (n=73)	*p*	Positive (n=94)	Negative (n=46)	*p*
AJCC Stage									
I	31	70	0.318	41	60	0.008	65	36	0.318
II to IV	16	23		26	13		29	10	
LN metastasis									
No	39	83	0.172	55	67	0.128	81	41	
Yes	8	10		12	6		13	5	0.790
Myometrial invasion									
<1/2 of myometrium	24	68	0.014	34	58	0.000	55	37	0.013
≥1/2 of myometrium	23	25		33	15		39	9	
Histologic grade									
1	18	31	0.578	16	33	0.013	26	23	0.014
2 and 3	29	62		51	40		68	23	
Overall survival									
Alive	30	84	0.000	51	63	0.134	74	40	0.355
DOD	17	9		16	10		20	6	

HIF-1α: hypoxia-inducible factor 1α; CA IX: carbonic anhydrase 9; GLUT-1: glucose transporter-1.

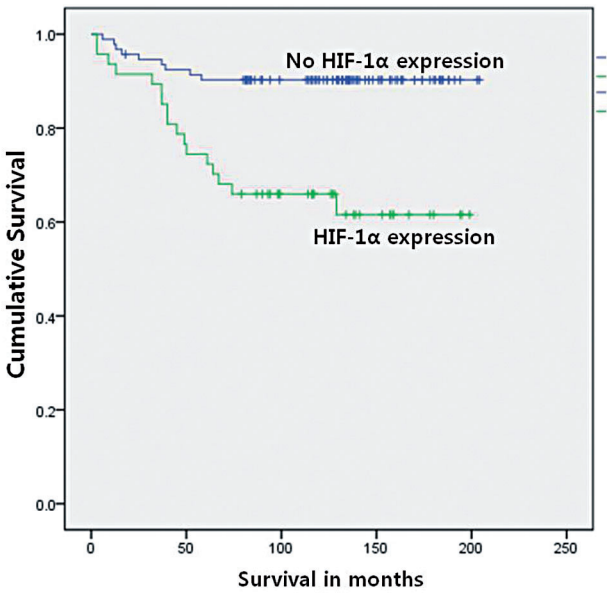

Figure 2. — Association of immunohistochemical staining for HIF-1α with overall survival of patients. The Kaplan-Meier survival curves show significant poor prognosis in patients with HIF-1α expression ($p = 0.000$).

Table 3. — *Multivariate analysis of prognostic factors for OS in EC.*

Variables	Grouping	*p*	Ratio of risk	95% of CI
FIGO Stage	I *vs*. II - IV	0.006	0.280	0.113-0.691
Myometrial invasion	< 1/2 *vs*. ≥ 1/2	0.222	0.597	0.262-1.365
LN metastasis	Absent *vs*. present	0.201	0.540	0.210-1.389
Histologic grade	1 vs. 2.2	0.044	0.329	0.111-0.973
HIF-1α expression	No *vs*. yes	0.001	0.241	0.105-0.552

Ozbudak *et al*. [18] observed that HIF-1α was increasingly expressed from early stages through advance stages of endometrioid adenocarcinoma, paralleled by activation of its downstream genes such as GLUT-1, VEGF. and increased angiogenesis. They suggested that these results highlighted the importance of hypoxia and related pathways in progression of endometrial carcinoma.

This study aimed to investigate the expression patterns of multiple hypoxic markers and their prognostic significance in EC using immunohistochemistry. Approaches to immunohistochemical evaluation for the scoring of hypoxic markers vary and may be complicated; simple and commonly used criteria were selected for use in this study in order to improve the reliability and consistency of interpretation. The expression of HIF-1α, CA IX, and GLUT-1 was observed to be significantly associated with deep myometrial invasion. The expression of HIF-1α was also associated with shorter OS. Furthermore, HIF-1α expression exhibited independent prognostic significance.

The various HIF-1α expression patterns have different prognostic implications in certain types of cancer. In breast cancer, patients with diffuse HIF-1α staining had a significantly better prognosis than patients with perinecrotically overexpressed HIF-1α [22]. Seeber *et al.* [23] suggested that perinecrotic HIF-1α expression was significantly associated with a shorter disease-free survival compared with diffuse HIF-1α expres-

sion in endometrioid endometrial carcinoma. The results of the current study revealed a difference in the expression of HIF-1α, CA IX and GLUT-1 according to myometrial invasion. The rate of expression of these molecules was significantly higher in cases showing deep myometrial invasion. HRFs are associated with invasiveness of tumor cells.

Hypoxic malignant cells are more resistant to radiotherapy and chemotherapy. In advanced stage ovarian carcinoma, GLUT-1 expression has been reported to be an independent prognostic factor of response to chemotherapy [24]. CA IX may also be an important marker in the prediction of drug responsiveness in tongue cancer chemotherapy [25]. Numerous studies have investigated the selective application of new treatment modalities based on targeting tumor hypoxia [26, 27]. reporting that hypoxic markers, including HIF-1α and CA IX, may be specific and favorable therapeutic targets. The present study demonstrated that the expression of these molecules was common in EC. HIF-1α, CA IX, and GLUT-1 may therefore be useful markers to indicate aggressive phenotypes and predict prognosis, and are also potent therapeutic targets.

In conclusion, the expression of hypoxic markers, including HIF-1α, CA IX, and GLUT-1 is common in patients with EC and is associated with tumor progression. In particular CA IX expression showed the significant association of their expression with higher histological grade and advanced tumor stage. In addition, the results suggest that HIF-1α expression is an independent unfavorable prognostic factor in EC. Additional investigation of hypoxic markers, including HIF-1α, as biomarkers of aggressive tumor behavior and as novel therapeutic targets, is warranted.

Acknowledgements

This study was supported by Medical Research Institute Grant (grant no. 2011-27), Pusan National University Hospital and by a grant from the National R&D Program for Cancer Control, Ministry for Health, Welfare and Family affairs, Republic of Korea (0920050).

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