ORIGINAL

Prognostic significance of HIF-2α expression on tumor infiltrating macrophages in patients with uterine cervical cancer undergoing radiotherapy

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Abstract : Hypoxia-inducible factor (HIF)-2α, a basic helix-loop-helix (bHLH)-PAS protein, is the principal regulator of the hypoxic transcriptional response. An immunohistochemical study reported strong HIF-2α expression in the cytoplasm of tumor infiltrative macrophages (TIMs). Thus we assessed the expression of HIF-2α in human cervical cancer tissue before radiation therapy and its relationship to the clinical outcome. Seventy three patients with histologically proven primary advanced squamous cell carcinoma of the uterine cervix underwent radiotherapy in Tokushima University Hospital after biopsy specimens were taken. Among 73 specimens stained for HIF-2α, 53 (72.6%) exhibited HIF-2α immunoreactivity in the TIMs. In only 5 of 73 cases, HIF-2α immunoreactivity was observed in the nuclei of tumor cells. The HIF-2α positive cell count ratio in TIMs was associated with disease-free survival (DFS) with the worst DFS (p=0.024) being in cases in the group with a high positive cell count ratio. A high HIF-2α positive cell count ratio in TIMs increased the risk of local recurrence (p=0.0142). These findings might suggest that the ratio of the HIF-2α positive cell in TIMs may be a new predictive indicator for prognosis before radiation therapy for uterine cervical cancer. J. Med. Invest. 55 : 78-86, February, 2008

Keywords **:** *hypoxia, HIF-2 alpha, macrophage, uterine cervical cancer*

INTRODUCTION

Hypoxia is an important factor in the progression of solid tumors and has been associated with various indicators of tumor angiogenesis as well as metastasis. The presence of widespread hypoxia in tumors has been associated with reduced survival following radiotherapy, surgery and chemotherapy. In radiotherapy, for example, hypoxic tumor cells are significantly less responsive to radiotherapy than their well-oxygenated counterparts because the oxygen-derived free radicals needed to potentiate ionizing radiation-induced DNA damage are reduced or absent in hypoxic cells (1). In a number of human malignant tumors, hypoxia is associated with poor outcome irrespective of the treatment modality used (2-5).

HIF-1 and HIF-2 are two closely related protein complexes that activate transcription of target genes in response to hypoxia. HIF-1 consists of a heterodi-

Received for publication October 24, 2007 ; accepted December 11, 2007.

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mer of HIF-1 α and HIF-1β, identical to the previously identified aryl hydrocarbon nuclear translocator (ARNT). Both are members of a family of transcription factors, termed bHLH/PAS proteins, which control a variety of critical embryogenic and physiological events. An alternative dimerization partner for ARNT, which also transactivates genes via HIF DNA recognition sites, has been identified and termed endothelial PAS domain protein 1 (EPAS-1), HIF-1α-like factor (HLF), and mouse HIF-related factor (HRF). In keeping with its functional homology with HIF-1α, this protein has been termed HIF-2α. HIF-2 consists of a heterodimer of HIF-2α and HIF-1β. Hypoxia, or genetic alterations of the hypoxia signalling cascade (6, 7) leading to the constitutive expression of HIF, could promote intense and chaotic neovascularization that facilitates tumour spread. It has now been firmly established that HIF has important roles in tumour progression. Several immunohistochemical analyses have indicated that HIF-1α and HIF-2α are overexpressed in primary and metastatic human cancers, and that the level of expression, either as a result of tumour hypoxia or genetic alterations, is correlated with tumour angiogenesis and patient mortality (7, 8).

HIF-1α and HIF-2α protein expression was mainly observed in nuclei of tumor cells in various human cancer cells. Talks, *et al*. reported strong HIF-2α expression in the cytoplasm of tumor infiltrative macrophages (TIMs) (9). To our knowledge, there is no published report of HIF-2α expression in TIMs in human uterine cervical cancer. Therefore, we assessed the expression of HIF-2α in human cervical cancer and the relationship between its expression and clinical outcomes.

MATERIALS AND METHODS

Patients

A retrospective analysis was done on all records of patients with histologically proven primary advanced squamous cell carcinoma of the cervix in the Department of Radiology, Tokushima University Hospital. Between December 1992 and March 2002, 73 patients with primary advanced squamous cell carcinoma of the cervix underwent radiotherapy. The clinical stage distribution according to the International Federation of Gynecology and Obstetrics (FIGO) criteria (10) was as follows : Stage IB = 3 (4.1%), Stage IIA = 3 (4.1%), Stage IIB = 16 (21.9%), Stage IIIA = 1 (1.4%), Stage IIIB = 35 (50%), Stage IVA = 7 (9.6%) and Stage IVB = 8 (11.0%). Patients were staged by use of chest radiography, intravenous pyelography (IVP), blood chemistry, cystoscopy, and rectosigmoidoscopy. Table 1 summarizes patient characteristics. Follow up and survival information was obtained from hospital records.

All patients had biopsy specimens taken pretreatment from the exposed tumor on the cervix of the uterus. All samples were immediately formalin-fixed and embedded in paraffin.

Table 1. Patients characteristics and treatment methods

Number of patients		73
Age range (median, years old)		42-93, (70)
Follow up period, range (median, month)		1-114, (30)
Histological classification	Squamouscell carcinoma	72
Stage (FIGO 1994)	Ib	3
	IIa	3
	IIb	16
	IIIa	1
	IIIb	35
	IVa	7
	IVb	8
Brachytherapy	LDR	48
	HDR	25
Concurrent chemotherapy	BOAI	14
	Weekly CDDP	5
	None	54
Maintenance chemotherapy	With	29
	Without	44

Radiotherapy Technique

For patients with primary advanced carcinoma of the cervix, radiotherapy consisting of a combination of intracavitary brachytherapy and external beam irradiation has been used in our department. Before August 1997, low dose rate intracavity brachytherapy (LDR) was delivered with a Cs-137 source. From September 1997, high dose rate intracavity brachytherapy (HDR) was delivered with an Ir-192 source.

External radiation therapy was performed with 6 MV X-rays using the anterior and posterior parallel opposing field technique. Five fractions weekly, with 1.8 or 2.0 Gy per fraction, were delivered to the midplane of the pelvis. The area of external radiation therapy included both the primary tumor and the regional lymph nodes. The fraction size was 1.8-2 Gy and total dose to the whole pelvis was 36-52 Gy (median 48 Gy) in the LDR group with midline shielding at 15-40 Gy (median 38 Gy), whereas in the HDR group the fraction size was 1.8-2 Gy and the total 40-50 Gy (median 45 Gy), with midline shielding at 20-45 Gy (median 43 Gy).

In the LDR group, we delivered the intracavitary irradiation either before or after external radiation therapy using a T.A.O manual afterloading application (11) with a Cs-137 source. The patient received 44-55 Gy (median 48 Gy) to point A with the external radiation therapy being designed to bring the dose to a total of 50-70 Gy through use of a midline shield. In the HDR group, intracavitary irradiation was delivered after external irradiation using a Modified Manchester Applicator, an Ir-192 source with a remotely controlled after-loading system (RALS) without general anesthesia. Once per week, at 6 Gy per fraction, a total of 12-30 Gy was delivered to point A.

Adjuvant therapy

Fourteen patients with locally advanced cancer received intra-arterial chemotherapy using the balloon occluded arterial infusion (BOAI) method and 5 patients received weekly cisplatin (CDDP) infusion as concurrent chemotherapy. A total of 29 patients underwent oral administration of fluorouracil or tegafur-uracil as maintenance chemotherapy. Maintenance chemotherapy was performed continuously for two years following radiation therapy.

Immunohistochemical study

A mouse monoclonal antibody (Mab), anti-EPAS-1/HIF-2α (EP190b ; Novus Biologicals, Littleton, CO, USA), was generated and characterized as reported previously (9, 12). Immunostaining was performed according to the Labeled StreptAvidin-Biotin (LSAB) method (Dako, Carpinteria, CA, USA), using a streptavidin-biotin-horseradish peroxidase complex. Briefly, deparaffinized and rehydrated sections were treated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature to block endogenous peroxidase activity. Antigen retrieval was achieved by a 750 W microwave in 10 mM citrate buffer (pH 6.0) for 15 min. After blocking with 10% goat serum in PBS, sections were incubated with the first antibody, and the reaction was carried out at 4°C overnight. All initial antibodies used for this study were tested for optimal dilutions, and a moderate dilution was determined for the best differentiation of tumor samples. We used 1 : 400-diluted EPAS-1 Mab (1.125 μg/ml). For immunohistochemical detection of macrophages, dilution of the Mabs anti-human CD68 (KP1, Novus Biologicals, Littleton, CO, USA) was done at 1 : 800. Reactions were visualized using the 3,3'-diaminobenidine substrate chromogen system (DAB) (K3468, Dako, Carpinteria, CA, USA). All IHC slides were counterstained by Mayer's hematoxylin.

Quantification of the Immunohistochemical study

A quantification method for the TIMs and HIF-2α in TIMs was based on a previous study (13, 14). To summarize, for focal macrophage quantification, all slides were first observed at low power magnification (×10) to select three areas of the highest concentration of CD68. The numbers of positively stained cells in the three areas were counted at high power magnification (×200). The average number of stained cells in the three 'hotspot' areas was considered as the CD68 count. The number of HIF-2α positively stained cells in the same area of the three hotspots was counted at high power magnification (×200). The average count was considered as the HIF-2α count. The average of the HIF-2α count was divided by the average of the CD68 count, and the HIF-2α/CD68 ratio was obtained.

Assessment was performed in blinded fashion and independently by two investigators (T. K., A. K.). We used the average data from the two investigators. Conflicting scores were resolved by discussion over the microscope.

Statistical analysis

A computer software Stat View-J 5.0 statistical

package (Abacus Concepts, Berkeley, CA, USA) was used for all statistical analysis and generation of survival curves. χ^2 tests examined the relationships between categorical tumor variables. Spearman-rank correlations were used to investigate relationships between continuous patient and tumor variables. Survival curves were plotted using the method of Kaplan and Meier, and the Log-rank test was used to evaluate differences between life tables. P values less than 0.05 were considered significant.

RESULTS

HIF-2α expression

In only 5 of 73 samples, HIF-2α immunoreactivity was observed in nuclei of tumor cells. All of these expressions were observed to be very weak at a high power field. No tumor cell contained immunoreactive cytoplasm (Fig. 1).

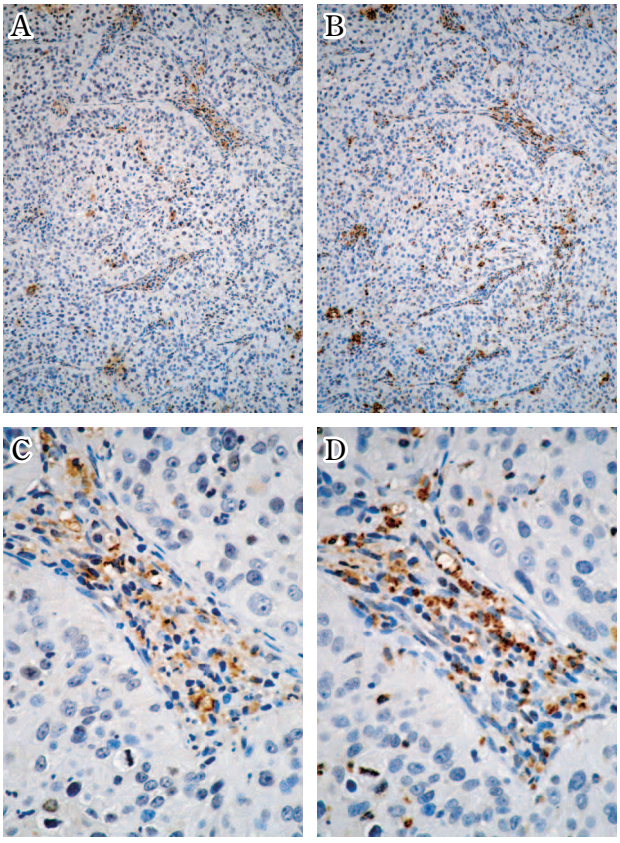

Fig. 1. Immunohistochemical study of HIF-2α and CD68 at human invasive uterine cervical cancer (squamous cell carcinoma) On (A) HIF-2α and (B) CD68 in low power field (×10), the uterine cervical squamous carcinoma show common positive cells observed in mainly interstitial tissue. On (C) HIF-2α and (D) CD68 in high power field (×200), both immunoreactivity was detected in common mononuclear cells that are distributed mainly in interstitial tissue and slightly in tumor parenchyma. In this case, a few tumor cells show HIF-2α immunoreactivity in nuclei.

CD68 immunoreactivity was detected in mononuclear cells that were distributed mainly in the interstitial tissue and slightly in the tumor parenchyma. These mononuclear cells were identified as TIMs (Fig. 1). HIF-2α immunoreactivity in colocalization with CD68 immunoreactive cells was found in 53 (72.6%) of the 73 samples. All the positive HIF-2α immunoreactivity in interstitial cells considered as TIMs was in the cytoplasm and was even stronger than that in tumor cells. The number of HIF-2α counts in 73 samples ranged from 0 to 79 with a median value of 14 (mean=18.7, SD=16.8), which was used as the cutoff point for categorical analysis. A group of 39 fell into the high HIF-2α count category (53%). The number of CD68 counts in 73 samples ranged from 0 to 169 with a median value of 57 (mean=62.4, SD=35.1). The high CD68 count group contained 38 samples (52%) using the cut-off point of 57. A positive correlation was found between increasing CD68 count (as a continuous variable) and increasing HIF-2α count (Spearman Rho=0.54, $p<0.0001$). The HIF-2α/CD68 ratio ranged from 0% to 100% with a median value of 23.2% (mean=29.2%, SD=25.7).

HIF-2α expression and clinical futures

In this study, the cumulative overall 5-year local control rate according to stage was 100% in stage I, 89% in stage II, 78% in stage III, 43% in stage IVa and 25% in stage IVb. The cause-specific 5-year survival rate was 100% in stage I, 79% in stage II, 58% in stage III, 43% in stage IVa and 13% in stage IVb. These data show similar results to previous reports on radiotherapy outcomes of uterine cervical cancer from other institutes (15-18). Table 2 shows the dis-

Table 2. Distribution of HIF-2α, CD68 and HIF-2α/CD68 in FIGO classification

		FIGO classification				
	n	I	II	III	IVa	IVb
HIF-2α counts						
(median = 14/HPF)						
HIF-2α < 14	34	3	8	17	4	2
HIF-2α ≧ 14	39	0	11	19	3	6
CD68 counts						
(median = 57/HPF)						
CD68 < 57	35	2	11	16	3	3
CD68 ≧ 57	38	1	8	20	4	5
HIF-2α / CD68 ratio						
(median = 23.2%)						
HIF-2α/CD68 < 23.2	35	3	7	21	2	2
HIF-2α/CD68 ≧ 23.2	38	0	12	15	5	6

tribution of HIF-2α count, CD68 count and HIF-2α/CD68 ratio in the FIGO classification. There were relatively balanced distributions of classified HIF-2α count, CD68 count and HIF-2α/CD68 ratio in the FIGO classification.

The HIF-2α count, CD68 count and HIF-2α/CD68 ratio were compared with DFS and cause-specific survival (CSS). The HIF-2α count and CD68 count failed to achieve significance for either DFS or CSS (Fig. 2 and 3). Only the HIF-2α/CD68 ratio was associated with DFS (p=0.024) with cases in the high category group having the worst DFS (Fig. 4). However the HIF-2α/CD68 ratio was not associated with CSS. The distribution of the HIF-2α/CD68 ratio is shown in Table 3. There was no correlation between the HIF-2α/CD68 ratio and age, con-

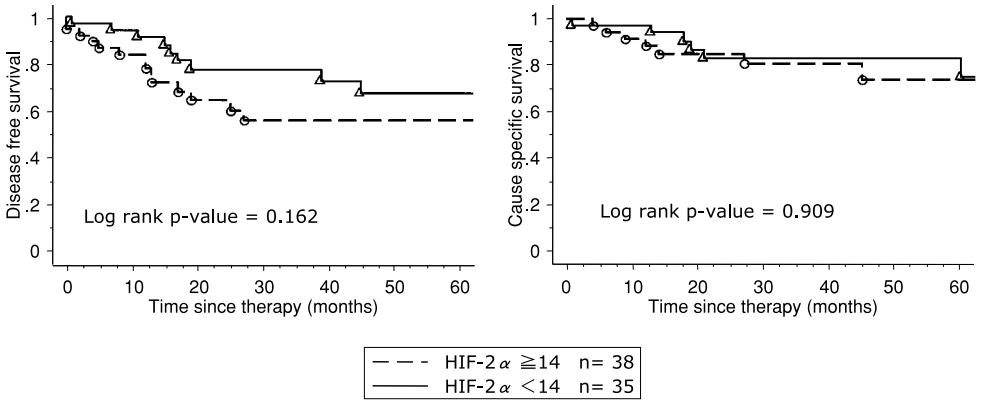

Fig. 2. Kaplan-Meier curves of DFS and CSS based on HIF-2α expression in TIMs

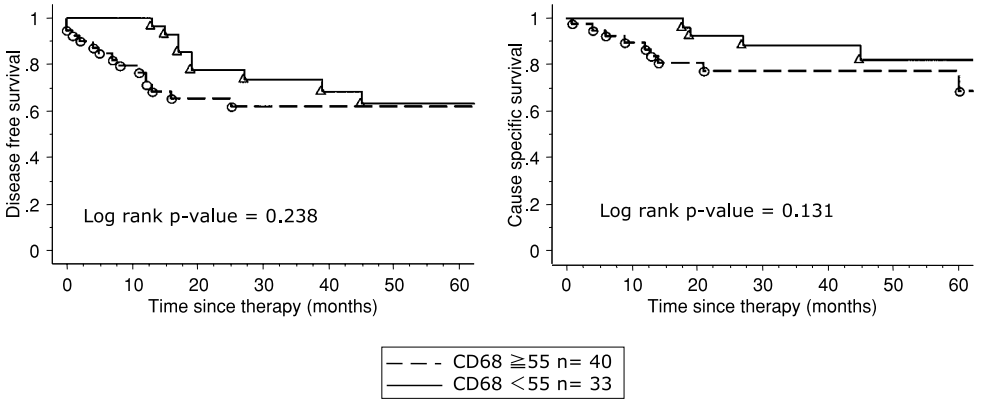

Fig. 3. Kaplan-Meier curves of DFS and CSS based on CD68 expression in TIMs

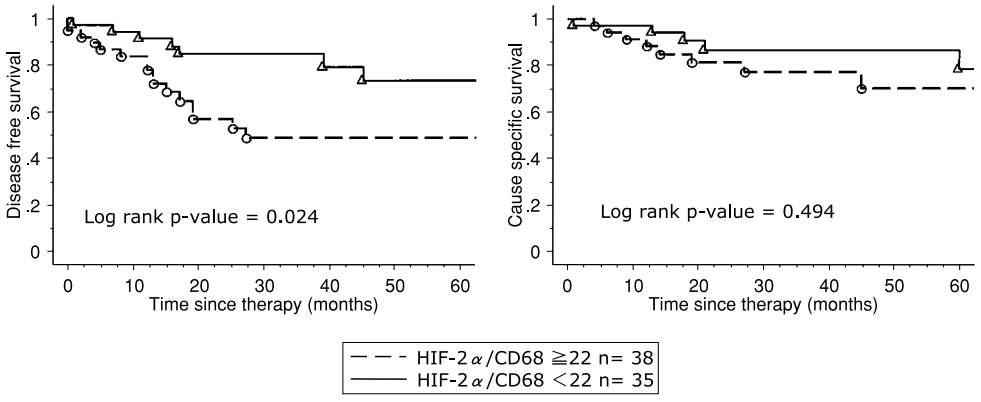

Fig. 4. Kaplan-Meier curves of DFS and CSS based on HIF-2α/CD68 ratio

Table 3. Distribution of HIF-2α/CD68 ratio

		Over all (n=73)	HIF-2α/CD68 ratio (%) <23.2 (n= 35)	HIF-2α/CD68 ratio (%) ≧23.2 (n= 38)	P value
Age (mean±SD)		70.3±11.2	71.5±12.0	70.8±10.8	p=0.794
Concurrent chemotherapy	With	19	11	8	p=0.317
	without	54	24	30	
Brachytherapy	LDR	48	25	23	p=0.227
	HDR	25	10	15	
Maintenance chemotherapy	With	29	15	14	p=0.590
	without	44	20	24	
Pretreatment hemoglobin level, mean±SD (/mm^3)		11.4±1.9	11.1±2.1	11.6±1.8	p=1.094
CD68 count, mean±SD(/HPF)		61.5±35.6	58.2±39.7	64.6±31.6	p=0.437

current or maintenance chemotherapy, brachytherapy method, pretreatment hemoglobin level or CD68 count. HIF-2α immunoreactivity in the nuclei of tumor cells had no relationship with DFS or CSS. (Data not shown)

In 19 of 73 patients tumors have recurred during our observation period. The high HIF-2α/CD68 ratio group had a significantly increased risk of local recurrence (p=0.0142) (Table 4).

Table 4. Treatment failure pattern

	HIF-2α/CD68 ratio (%) <23.2 (n= 35)	HIF-2α/CD68 ratio (%) ≧23.2 (n= 38)
Local only	2	7
Distant (With local)	5 (1)	5 (1)
Overall	7	12

DISCUSSION

To our knowledge, this is the first report about HIF-2α in squamous cell carcinoma of the uterine cervix. Major findings in this survey were that most of the HIF-2α immunoreactivity in squamous cell carcinoma of the uterine cervix was detected in TIMs and the percentage of HIF-2α immunoreactivity in TIMs significantly correlated with DFS.

It is well established that, when exposed to lowered levels of oxygen, tissues compensate in a variety of ways, ranging from systemic adjustments caused by increased erythropoietin production to tissue-specific effects of increased VEGF expression and the largely cellular effects of increased glycolysis (19). All of these adaptations to hypoxia are regulated wholly or in part by the HIF complex. The oxygen-regulated components of this complex are the HIF-α subunits. To date, three members of the HIF-α family have been cloned : HIF-1α, HIF-2α, and HIF-3α. Of the three HIF-α subunits, the most extensively characterized subunit regarding function is HIF-1α. Its hypoxia-induced stabilization and the following induction of a number of target genes seems to be a general response in most, if not all, cells. In addition, these proteins have at least some unique functions as revealed by their different target genes (20-24). By contrast, HIF-3α is involved in downregulation of the hypoxic response via an alternatively spliced transcription factor, which may function as an inhibitor of HIF-1α (25, 26).

HIF-1α and HIF-2α expression are a significant prognostic factor of various cancers treated by radiotherapy (12, 27-30). In uterine cervical cancer, some papers (31-34) reported that overexpression of HIF-1α in pretreatment uterine cervical cancer cells served as a predictive marker for poor prognosis after treatment by radiotherapy. However, there has been no study examining the relationship between the expression of HIF-2α and uterine cervical cancer. In this study, weak HIF-2α immunoreactivity in tumor cells was observed in only five biopsy sections and was not associated with prognosis. This difference in immunoreactivity between HIF-1α and HIF-2α in cervical cancer cell seems to indicate that there are individual variations in the hypoxic response. Findings relating to the role of TIMs in cervical cancers have been controversial. It has been noted that there are higher

macrophage counts in invasive carcinomas as opposed to cervical squamous intraepithelial lesions (35). In turn, squamous intraepithelial lesions contain more macrophages than the normal cervix (36). Davidson, *et al*. (37) looked at 75 cases of carcinoma, staining the tissue with CD68 and two endothelial markers, and evaluated the cases by light microscopy. Based on previous reports of an association between TIM density, increased angiogenesis, and poorer outcome in breast carcinoma (38), these authors sought to evaluate the same in cervical cancer. No correlation was found between TIM count and tumor stage, grade, or survival, nor with microvessel count.

Leek, *et al*. reported that HIF-2α expression in TIMs was not associated with DFS but was marginally associated with overall survival in cases in the high expression group which had worse survival in invasive breast cancer (39). To our knowledge, this was the only report which mentioned a correlation between HIF-2α expression in TIMs and clinical outcome. However in this study, HIF-2α expression in TIMs was associated with DFS. Additionally there was a significant tendency for an increased risk of local recurrence in cases with a high HIF-2α expression in the TIM group. This recurrence pattern suggests that the role of HIF-2α expression in TIMs is more important in regional tumor development than in distant metastasis. It seems that HIF-2α secreted by TIMs is a very important key factor in tumor survival. Thus if we can control TIM behavior, it must then contribute to remission of the tumor.

In conclusion, we evaluated HIF-2α expression in uterine cervical cancer. Most of the HIF-2α expression was observed in TIMs and a high HIF-2α expression ratio in TIMs was associated with DFS and local recurrence. These results demonstrated that macrophages recruited into tumor tissue play a role in promoting tumor growth and that HIF-2α expression in TIMs is an important prognostic factor, especially for predicting future local recurrence after radiotherapy in patients with advanced uterine cervical cancer.

ACKNOWLEDGMENTS

We thank Ms. Noriko Amou, Department of Human Pathology, Institute of Health Biosciences, The University of Tokushima Graduate School, for excellent advice for Immunohistochemical technique.

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