



Vasculogenic mimicry and hypoxia-inducible factor-1 α expression in cervical squamous cell carcinoma

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ABSTRACT. In this study, the existence of vasculogenic mimicry (VM) in cervical squamous cell carcinoma was investigated. To this end, the relationship between hypoxia-inducible factor 1 α (HIF-1 α) and the development, infiltration, and metastasis of cervical squamous cell carcinoma was studied. Between January 2010 and December 2010, 67 human cervical squamous carcinoma tissue samples were collected and stained by CD34/periodic acid-Schiff double staining to detect the existence of VM. HIF-1 α expression was analyzed by immunohistochemistry. The relationship between VM and HIF-1 α was also analyzed. Normal cervical tissues (20 cases) from patients who had uterine surgeries in the same period were collected as controls. In the cervical squamous carcinoma tissues, positive rates of VM and HIF-1 α were 38.81% (26/67) and 64.18% (43/67), respectively. This was significantly higher than those in the normal cervical tissues [0 (0/20); $P < 0.05$]. VM rates in cervical squamous carcinoma tissues from patients with different pathological grades, Federation of Gynecology and Obstetrics (FIGO) stages, and lymph node

metastasis states were also significantly different ($P < 0.05$). In addition, significant differences in HIF-1 α positivity rates were observed among patients with varying tumor sizes and lymph node metastasis states ($P < 0.05$). Positive correlation was found between VM and HIF-1 α ($r = 0.339$, $P < 0.05$). To summarize, we found VM in cervical squamous carcinoma; high expression of HIF-1 α may promote VM formation, as well as cervical squamous cell infiltration and metastasis.

Key words: Cervical neoplasms; Cancer; Squamous cells; Hypoxia-inducible factor 1 α ; Immunohistochemistry; Vasculogenic mimicry

INTRODUCTION

Adequate blood supply is essential for the growth, local infiltration, and distant metastasis of solid tumors. Existing studies have shown that 3 main avenues by which tumors receive blood supplies are via angiogenesis, mosaic vessels, and vasculogenic mimicry (VM) (Scully et al., 2012). VM is a new kind of tumor microcirculation model, which simulates morphological structure of endothelium-dependent vessels and provides nutrition for tumors. It is found in a variety of solid tumors and is related to prognosis. Hypoxia is conducive to VM formation. Hypoxia-inducible factor 1 α (HIF-1 α) is a key transcriptional regulatory factor of the adaptive response under hypoxic microenvironments. It promotes the infiltration and metastasis of tumors by regulating the metabolism and proliferation of tumor cells and cell proliferation, thus inducing tumor angiogenesis. In this study, we aimed to detect the existence of VM in cervical cancer, as well as to examine the expression of HIF-1 α in cervical squamous carcinoma tissues. Furthermore, the role of VM and HIF-1 α in the development, infiltration, and metastasis of cervical squamous cell carcinoma was also investigated.

MATERIAL AND METHODS

Materials

Patients and tissue specimens

Between January 2010 and December 2010, cervical squamous carcinoma tissue specimens from 67 cases were excised during surgery. Diagnosis was confirmed via pathological analysis at the Affiliated Hospital of Luzhou Medical College. All patients had intact medical records, and it was the primary cervical cancer. Patient age ranged between 24 to 65 years (average, 43.67 ± 8.14 years); the patients did not receive any radiotherapy or chemotherapy before surgery. Based on the criteria set by the Federation of Gynecology and Obstetrics (FIGO, 2000) stages, 36 cases were classified as stage I cancer, and 31 cases were classified as stage II cancer. There were 27 patients with tumors greater than 4 cm in diameter, and 40 patients with tumors less than 4 cm in diameter. According to the histological classification standards of WHO, 21 cases were considered keratinizing, while the remaining 46 cases were non-keratinizing. In addition, 29 patients also demonstrated lymph node metastasis. During the same period, 20 cases of normal cervical tissue from patients who had uterine surgeries were

collected as controls. All specimens were fixed with 4% neutral formalin, embedded in paraffin, and serially sectioned into 4- μ m thick sections.

Main reagents

Mouse anti-human CD34 monoclonal antibody was purchased from Genentech, Inc. (Shanghai, China), and rabbit anti-human HIF-1 α polyclonal antibody was purchased from Bioworld Technology, Inc. (St. Louis Park, MN, USA). Envision kits were bought from Dako (Santa Clara, CA, USA), and periodic acid-Schiff (PAS) dye was prepared according to Pathological Techniques (Wang, 2000).

Methods

CD34/PAS double staining

Paraffin sections were dewaxed and repaired under high pressure. Endogenous peroxidase was eliminated with 3% H₂O₂, and CD34 monoclonal antibody (1:100) was added onto the sections and incubated overnight. Manufacturer instructions from the Envision immunohistochemistry kits were strictly followed. After the DAB chromogenic reaction, PAS procedures were followed. Hematoxylin staining and gradient dehydration were carried out prior to mounting with neutral gum.

Immunohistochemical staining

Staining was performed as per instructions from Immunohistochemistry Envision. Paraffin sections were dewaxed and repaired under high pressure. Endogenous peroxidase was eliminated with 3% H₂O₂. HIF-1 α polyclonal antibody (1:100) was added onto the sections and incubated overnight. Envision secondary antibody was then added and incubated at 37°C for 2 h. DAB chromogenic reaction, hematoxylin staining, and gradient dehydration were carried out prior to mounting with neutral gum.

Methods used for analysis of immunohistochemistry

All immunohistochemistry results were blindly analyzed by 2 physicians, and at least 10 non-repeated images from a section at high power (400X) were selected to determine the results. Based on previous studies (Wang, 2000), we adopted a semi-quantitative method that divided the staining intensity into 4 levels and scored them as follows: no staining, 0 point; yellow, 1 point; brown-yellow, 2 points; tan, 3 points. We also divided cells into 4 grades according to the proportion of positively stained cells. Positive cells <10%, 0 points; 10-40% positive cells, 1 point; 40-70% positive cells, 2 points; \geq 70% positive cells, 3 points. The sum of the 2 scores was used to estimate the results. A sum of 0-1 points was recorded as (-), 2 points as (+), 3-4 points as (+ +), and 5-6 points as (+ + +). During statistical analysis, (-) denoted negative cells, and the rest represented positive cells.

VM judgment

In each section, 10 non-repeated light microscope images were randomly chosen for

counting. The vascular endothelium was identified by positive CD34 staining. CD34-negative lumens had reddish (PAS positive) extracellular matrix on the outside, as well as walls composed of tumor cells, which was verified by hematoxylin-eosin staining (HE staining). These structures were classified as VM positive.

Statistical analysis

The SPSS13.0 software was used for statistical analysis, and data are reported as rates or proportion. All comparisons were carried out using the χ^2 test. Correlational analyses were performed using Spearman correlation, with $P < 0.05$ being considered as significant.

RESULTS

Presence of VM in cervical squamous cell carcinoma tissue

HE staining results showed tubular structures composed of tumor cells with erythrocytes inside parts of the lumens. However, tumor necrosis and inflammation cells were not detected. The results of CD34/PAS double staining showed the presence of tubular, PAS-positive structures forming long strips with irregular or annular shapes, which separated tumor cells and erythrocytes. Furthermore, tubular structures were CD34 negative, indicating a lack of erythrocytes. VM mainly appeared in avascular or hypovascular areas, which were absent in euangiotic areas. Among the 67 cases of cervical squamous carcinoma tissue samples, VM was detected in 26 cases with a positive rate of 38.81% (26/67 cases). In the 20 healthy cervical tissue samples, no VM was observed (0/20 cases).

HIF-1 α expression in cervical squamous carcinoma tissue

HIF-1 α was mainly expressed in the cytoplasm and partly in the nuclei. Among the 67 cases of cervical squamous carcinoma tissue samples, 43 patients had positive signals (64.18%). However, HIF-1 α was not expressed in the 20 healthy cervical tissues (0/20 cases).

Relationship between VM, HIF-1 α , and clinical pathologic features in the cervical squamous carcinoma tissue

The presence of VM in cervical squamous carcinoma tissues from patients of different ages was compared; the findings were similar ($P > 0.05$). Similarly, there were also no statistical differences in the presence of VM among patients of different FIGO stages ($P > 0.05$). However, as shown in Table 1, when the appearance of VM was compared among patients with different tumor size and lymph node metastasis conditions, there were significant differences between the groups ($P < 0.05$).

Relationship between VM and HIF-1 α in cervical squamous cell carcinoma

The presence of HIF-1 α in VM-positive cervical squamous cell carcinoma tissues was 80.77% (22/26), while that in VM-negative cervical squamous cell carcinoma tissues was 51.22% (21/41). The results indicated that VM in cervical squamous carcinoma tissues was positively correlated with HIF-1 α expression ($r = 0.339$, $P = 0.005$; Table 2).

Table 1. Relationship between VM, HIF-1 α and the clinical pathologic features in cervical squamous carcinoma tissue [N (%)].

Clinical pathologic feature	N	VM positive	Z	P value	HIF-1 α positive	Z	P value
Age (years)							
<40	24	10 (41.67)	0.129	0.720	15 (62.50)	0.046	0.830
\geq 40	43	16 (37.21)			28 (65.12)		
Tumor size (cm)							
\leq 4	40	15 (37.50)	0.071	0.789	21 (52.50)	5.889	0.015
>4	27	11 (40.74)			22 (81.48)		
Pathological grading							
Keratinizing	21	4 (19.05)	5.028	0.025	12 (57.14)	0.217	0.642
Non-keratinizing	46	22 (47.83)			31 (67.39)		
FIGO stages							
I	36	8 (22.22)	9.011	0.003	20 (55.56)	2.517	0.113
II	31	18 (58.06)			23 (74.19)		
Lymph node metastasis							
None	38	7 (18.42)	15.363	0.000	19 (50.00)	7.678	0.006
Yes	29	19 (65.52)			24 (82.76)		

Table 2. Relationship between VM and HIF-1 α in cervical squamous cell carcinoma (N).

VM in cervical squamous cell carcinoma tissues	HIF-1 α		Sum
	Positive	Negative	
Positive	22	4	26
Negative	21	20	41
Sum	43	24	67

DISCUSSION

Cervical carcinoma is one of the most common malignant tumors in the female reproductive system with a morbidity rate of 12.96/100,000 (Ying et al., 2013). With the use of cervical smear cytological examinations, the detection rate of early-stage cervical carcinoma and precancerous lesions has increased. However, changes in living habits and lifestyles have increased the morbidity of invasive cervical carcinoma, which are increasingly observed in younger patients. Cervical squamous cell carcinoma is the most common cervical histological type with poor prognosis. Thus, studies on the mechanisms underlying its development, infiltration, and metastasis can have a positive significance for clinical treatment of cervical carcinoma.

Tumors have the intrinsic characteristic of inducing neovascularity, which provides the necessary nutrients for its growth. Furthermore, the nascent tumor vascular walls are immature, and tumor cells can migrate into the vessel lumen, resulting in tumor infiltration and metastasis (Chai et al., 2012). According to traditional theories, blood supply for tumors is provided by these vessels. However, based on studies in uveal melanoma, Maniotis et al. (1999) found PAS-positive substances in tumors that formed tubular structures of uneven thickness. They presented as long strips with irregular or annular shapes, and were negative for endothelial cell markers such as CD34 and CD31, suggesting that these blood-supplying vessels lacked endothelial cells. As a result, the idea of a special blood supply called vasculogenic mimicry (VM) was put forth. VM was later discovered in various types of solid tumors, especially in highly malignant solid tumors (Lin et al., 2012; Lirdpramongkol et al., 2012; Tijeras-Raballand et al., 2014). In this study, HE stain-

ing also showed tubular structures composed of tumor cells with erythrocytes inside parts of the lumens. However, tumor necrosis and inflammatory cells were not found. Similar to the findings of previous studies, a PAS-positive substance negative for CD34 was also found. These PAS-positive structures were not observed in normal cervical tissues. VM mainly appeared in avascular or hypovascular areas, which were absent in euangiotic areas, indicating the existence of VM in cervical squamous cell carcinoma tissues. VM in cervical squamous cell carcinoma tissues was not correlated to the tumor size or age of patients, but it was correlated to FIGO stages, histological grades, and lymphatic metastasis, suggesting that VM may be an important indicator for cancer diagnosis.

Studies have shown that changes in the tumor microenvironment play an important role in tumor infiltration and metastasis, as well as in VM formation. Moreover, hypoxia is an important component of the tumor microenvironment (Francescone et al., 2012; Borsi et al., 2014). Tumor growth requires continuous supply of oxygen. However, oxygen diffuses slowly at a speed of 100-180 $\mu\text{m}/\text{s}$ in tissues, which does not meet the demands of the rapidly growing tumor and results in regional hypoxia (Xiao and Zheng, 2014). HIF-1 α is a recently discovered hypoxia marker (Yasuo et al., 2011). Under anoxic conditions, HIF-1 α is activated and regulates transcription of various downstream target genes and acts to maintain anaerobic metabolism of tumor cells. In addition, it also promotes neo-vascularization, thus enhancing tumor survival, growth, and metastasis (Osada et al., 2007). In this study, expression of HIF-1 α was noted in 64.18% (43/67) of cervical squamous cell carcinoma cases, which differed significantly from that of normal cervical tissues ($P < 0.05$). Moreover, it was not associated with age, histological differentiation, and FIGO stages of patients, but was correlated with lymph node state and tumor size, suggesting that HIF-1 α plays an important role in carcinogenesis, infiltration, and metastasis of cervical squamous cell carcinoma. In addition, correlation analysis showed that VM in cervical squamous cell carcinoma was positively correlated with HIF-1 α expression. It is possible that the rapid tumor growth results in insufficient blood supply inside tumor tissues. Prior to endothelium-dependent vessels ingrowth, VM develops as an alternative approach to alleviate the hypoxic state of tumors. Therefore, HIF-1 α may be important in VM formation.

In conclusion, VM was found in cervical squamous cell carcinoma tissues, and HIF-1 α expression may be important for VM formation. Therefore, joint detection of VM in cervical squamous cell carcinoma tissue and HIF-1 α has important implications for predicting the occurrence, development, and diagnosis of cervical cancer, and provides theoretical basis for new tumor treatments.

Conflicts of interest

The authors declare no conflict of interest.

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