RESEARCH ARTICLE

Expression of Endogenous Hypoxia Markers in Vulvar Squamous Cell Carcinoma

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Abstract

Objective: To investigate the expression of endogenous hypoxia-related markers identified as being involved in vulvar squamous cell carcinoma (VSCC). Methods: We performed immunohistochemical staining of hypoxia-inducible factor-1α (HIF-1α), glucose transporter-1 (GLUT-1), carbonic anhydrase 9 (CA-9) and vascular endothelial growth factor (VEGF), on tissue sections of 25 VSCC patients, 10 vulvar intraepithelial neoplasia (VIN) patients and 12 healthy controls. Results: HIF-1α expression was found in all sections, with no significant difference between controls, VIN and VSCC sections (all P<0.05). Glut-1 expression was found in 25% of control, 90% of VIN and 100% of VSCC sections. A significant difference between control and VIN or VSCC was observed (all P<0.05), while no difference was found between VIN and VSCC sections (P>0.05). CA-9 expression was negative in control sections, but it was found in 30% of VIN sections and 52% of VSCC sections with strong staining. Similarly, CA-9 expression also showed obvious differences between controls and VIN or VSCC sections (all P<0.05). However, there was no significant difference between VIN and VSCC (P>0.05). There were only 25% of control sections with weak VEGF expression, while strong staining was found in about 60% of VIN sections and 25% of VSCC sections (all P<0.05). In addition, a difference was also found between VIN and VSCC sections (P<0.05). Conclusion: Expression of endogenous hypoxia markers (HIF-1α, GLUT-1, CA-9 and VEGF) might be involved in the malignant progression of VSCC.

Keywords: Hypoxia - endogenous hypoxia marker - vulvar squamous cell carcinoma - vulvar intraepithelial neoplasia

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Introduction

Vulvar squamous cell carcinoma (VSCC) is the fourth most common type of gynecological cancer and affects the external female genitalia. It accounts for approximately 3~5% of all gynecological malignancies, with an incidence rate of 1~2/100000 (van de Nieuwenhof et al., 2011). VSCC share common risk factors with cervical cancer, such as increased number of sexual partners, younger age at first episode of sexual intercourse, abnormal papanicolaou smear, presence of genital warts, tobacco use, lichen sclerosus, diabetes, and human papillomavirus (HPV) infection (Tyring, 2003). It has been hypothesized that most of VSCC may be prevented by the recently introduced human papillomavirus 16/18 vaccines. However, the majority of VSCC develop in a background of lichen sclerosus (LS) and differentiated vulvar intraepithelial neoplasia (VIN), of which the exact etiopathogenesis is incompletely understood (van de Nieuwenhof et al., 2009).

Hypoxia is the result of an imbalance between oxygen delivery and oxygen consumption, which is thought to be one of the most important events during carcinoma progression, because it renders a more aggressive phenotype with increased invasiveness and proliferation, formation of metastases and poorer survival (Bussink et al., 2003; Seeber et al., 2011). Hypoxia is a powerful trigger for changes in gene expression and protein synthesis (Wenger, 2002). Overexpression of hypoxia-inducible factor (HIF) is found in common human cancers, and it is a positive factor in solid tumor growth and a negative prognostic factor for cancer patients (Birner et al., 2000; Semenza, 2012). The HIF-1α and HIF-2α can dimerize with the constitutively expressed HIF-1β subunit to bind to a hypoxia-responsive element to activate a wide array of genes, including those involved in anaerobic metabolism, cell cycle arrest, differentiation, stress adaptation, angiogenesis, and others (Coleman et al., 2002; Semenza, 2010). These can result in profound alterations on tumor and cellular phenotype, including an obvious

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role in angiogenesis by upregulation of angiogenic factors (Hua et al., 2006). Hypoxia is also a mediator of carcinogenesis, with mechanisms that include selection pressure, genomic instability, genomic heterogeneity, decreasing apoptotic potential, increasing angiogenesis, and a disorder microcirculation (Harris, 2002; Liao and Johnson, 2007; Mizukami et al., 2007). Therefore, HIF and its effector gene and protein seem to be excellent targets for innovative molecular therapeutics.

Protein members of the transcriptional response to hypoxia expressed in tumor tissue, such as HIF-1α, glucose transporter-1 (GLUT-1), carbonic anhydrase 9 (CA-9) and vascular endothelial growth factor (VEGF) are currently being discussed as "endogenous hypoxia-related markers" (Jonathan et al., 2006). A landmark study on the expression of HIF-1α protein in HeLa cells during chronic hypoxia reported detectable protein at 20% O2, a modest increase between 20% and 6%, a dramatic rise below 6% with a maximum at 0.5%, and a drop at 0%, attributed to an effect of anoxia on overall transcription and translation (Jiang et al., 1996). Immunohistochemical analysis of human cancer biopsies revealed increased levels (relative to surrounding normal tissue) of HIF-1α protein in various types of cancers and their metastases (Zhong et al., 1999; Krieg et al., 2000; Talks et al., 2000; Semenza, 2003). GLUT-1 is a representative member of the glucose transporter family and is widely distributed in normal tissues such as erythrocytes and endothelial cells at the blood-brain barrier (Yasuda et al., 2008). GLUT-1 is involved in the metabolic adaptation of the cell to hypoxia by increasing the intracellular glucose supply, which might be regulated by HIF-1α (Rademakers et al., 2011). CA-9, one of the 15 carbonic anhydrase isoforms in humans, which is exclusively transactivated by HIF-1α, has recently emerged as one of the most promising endogenous hypoxia-related markers of cellular hypoxia (Kaluz et al., 2009). Angiogenesis is an essential biological process not only in embryogenesis but also in the progression of a variety of major diseases such as cancer, diabetes and inflammation (Shibuya, 2001). VEGF family and its receptor system has been shown to be the fundamental regulator in the cell signaling of angiogenesis (Carmeliet and Jain, 2000; Ferrara, 2002). The HIF-1α is also a physiological regulator of VEGF expression (Linden et al., 2003). Increased expression of endogenous hypoxia markers have been demonstrated in various malignant tumors, including colorectal cancer, lung cancer, thyroid cancer, ovarian cancer and esophageal cancer, it has been reported that the of overexpression is related to a higher frequency of lymph node metastasis, extra-organ growth and poor prognosis (Osinsky et al., 2009). However, to date there are no data assessing endogenous hypoxia markers specifically for VSCC. To our knowledge, this is the first study to focus on the relationship between endogenous hypoxia markers and VSCC.

Materials and Methods

Patients materials

Criteria for VSCC patient participation in the study include: i) patients who had been diagnosed with VSCC and had undergone a resection at Qilu Hospital of Shandong University; ii) presence of involved lymph node or tumor that was accessible for microelectrode measurement; and iii) willingness to sign an informed consent approved by Medical Ethic and Human Research Committee of Shandong University, Jinan, China.

Three study groups were identified, including 25 VSCC patients diagnosed between 2002 and 2011 and two control groups consisting of 12 female population controls and 10 patients diagnosed with VIN in the same time period as the VSCC patients. All histological specimens of VSCC patients were reviewed by an experienced pathologist without access to any clinical information on the patients. The median age of VSCC patients was 63 years (range, 42-76 years), 43 years (range, 29–67 years) for VIN and 44 years (range, 28–58) for healthy controls, respectively. The clinical stage was classified according to the new International Federation of Gynecology and the Obstetrics (FIGO) classification from 2009 (Pecorelli, 2009). Sixteen (64%) VSCC patients were classified to stage I/II, and nine (36.0%) patient with stage III/IV. Radical surgery was performed in 19 (76%) of these cases and the remaining 6 (24%) patients received non-radical surgery. Postoperative therapy was administered to 13 VSCC patients including irradiation in 7 (28%) cases, and chemotherapy in six (24%) cases. Eight (32%) of the patients in this cohort died as a result of their vulvar cancer. All patients were followed up from the time of their confirmed diagnosis until death or 1 June, 2012. The median follow-up time was 76 months (range, 41 to 112 months).

For immunohistochemical evaluation, archival formalin-fixed, paraffin-embedded vulvar specimens collected. Vulvar specimens were obtained by punch biopsies. Samples of control tissue were obtained during vulvar biopsies or gynecological reconstructive surgery. Women who pregnant or breastfeeding, and subjects with a history of treatment with topical or systemic radiotherapy or chemotherapy were excluded from the study.

Lidocaine 2% was injected for anesthesia when all the samples were taken. Adrenalin was not used during the procedure. Hematoxylin-eosin (HE) stained slides from all cases were also reviewed by an experienced pathologist to reconfirm the histological diagnosis. Cases with ambiguous diagnoses were excluded.

Immunohistochemical procedure and evaluation

For immunohistochemical analysis, 3-μm sections were prepared. Each section was dewaxed using xylene, and hydrated by gradient alcohol. Antigen retrieval was performed using 1mm/L ethylene diamine tetra-acetic acid (EDTA) pH 6.0 for 15min at 95–100 °C for VEGF and in 0.01 mol/L citrate buffer (pH 6.0) at 100 °C for 45 min for HIF-1α and for 15 min for GLUT-1 and CA-9. After blocking with goat serum (only for staining for HIF-1α and GLUT-1), sections were individually incubated with monoclonal primary antibodies (Abcam, Cambridge, UK) to HIF-1α (dilution 1:3000), GLUT-1 (dilution 1:200), CA-9 (dilution 1:2000) and VEGF (dilution 1:35) overnight at 4 °C. After rinsing with phosphate-

buffered saline (PBS), sections were visualized with an avidin-biotin complex technology kit (Zymed, San Diego, CA, USA) for HIF-1α, GLUT-1 and CA-9, and a biotin-free horseradish peroxidase (HRP) polymer detection technology kit (GBI, Mukilteo, Washington, USA) for VEGF. Sections were counterstained with hematoxylin. Samples of glioblastoma multiforme, esophageal carcinoma, renal carcinoma and invasive breast cancer tissues served as positive controls for the four antibodies, respectively.

Staining was evaluated independently on coded slides by two observers. Nuclear staining of HIF-1α was considered positive, regardless of staining intensity (Jubb et al., 2004). Intact cell membrane staining of GLUT-1 and CA-9 in epidermal prickle cells and tumor cells were estimated. Staining intensity for GLUT-1 was scored on a 4-point scale (0 for no stained, 1 for <10% stained, 2 for 10-50% stained and 3 for 50-100% stained) as described by previous study (Tian et al., 2004). Staining intensity for CA-9 was scored on a 3-point scale (0 for <5% stained, 1 for <5-20% stained and 2 for >20% stained) (Choi et al., 2008). Fine cytoplasmic granular staining of VEGF in the epidermal prickle cells and tumor cells associated with microvessels was considered positive. The percentage of microvessels with immunopositive cells from the total number of microvessels in the papillary and reticular dermis was scored for the staining intensity of VEGF (1 for <25% of microvessels stained, 2 for 25-75% stained, 3 for >75% stained) (Davies et al., 2006). Adjustments were made for the apparent increased intensity of staining due to pigmentation in the basal layer by comparison with sections stained with hematoxylin only.

Statistical analysis

SPSS software (version 17.0; SPSS Inc., Chicago, IL, USA) was used for database management. A non-parametric (Mann–Whitney) test was used for analysis of ordinal categorical variable data. Correlations between numerical variables were obtained using Spearman's rank correlation. All statistical tests were two-tailed. P<0.05 was considered significant.

Results

Immunohistochemical staining of HIF-1α

All sections, including those of positive controls (glioblastoma multiforme, esophageal carcinoma, renal carcinoma and invasive breast cancer), found that HIF-1α expression occurred mostly in the cytoplasm, occasionally in the cytomembrane and rarely in nuclei. There was no significant difference in results for HIF-1α expression between healthy controls, VIN and VSCC patients (all P<0.05).

Immunohistochemical staining of GLUT-1

Glut-1 expression was negative or weak (score 0–1) in 75% of control sections (Figure 1a). Moderate to very strong expression (score 2–4) was found in about 90% of VIN sections (Figure 1b) and 100% of VSCC sections (Figure 1c). A significant difference between control and VIN or VSCC was found (P<0.05). But there was no difference between VIN and VSCC sections (P>0.05) (as shown in Table 1).

Table 1. Expression of GLUT-1, CA-9 and VEGF in Three Groups

Markers		Controls (n=12)	VIN (n=10)	VSCC (n=25)
GLUT-1				
Intensity score	0	5	0	0
	1	4	1	0
	2	2	5	5
	3	1	3	12
	4	0	1	8
CA-9				
Intensity score	0	12	7	12
	1	0	3	9
	2	0	0	4
VEGF				
Percentage score	0	2	0	0
	1	8	3	4
	2	2	6	6
	3	0	1	15

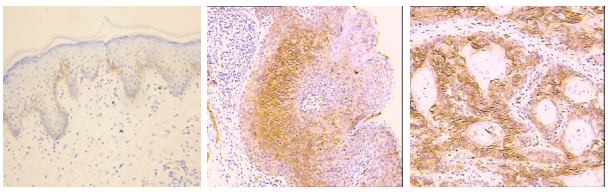

Figure 1. Immunohistochemical Staining of Glucose Transporter-1 (GLUT-1). (a) Control skin: Very weak staining sporadically expressed in basal cells and prickle cells (score = 0); (b) VIN tissue: Strong staining was seen in most basal cells and some prickle cells (score = 3); (c) VSCC tissue: Strong staining was seen in most basal cells and some prickle cells (score = 3)

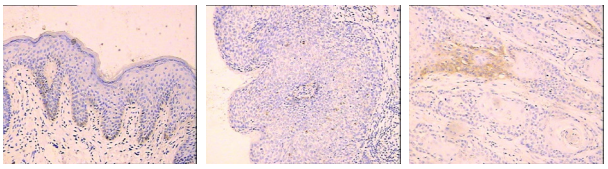

Figure 2. Immunohistochemical Staining of Carbonic Anhydrase 9 (CA-9). (a) Control skin: Very weak staining sporadically expressed in basal cells and prickle cells (score = 0); (b) VIN tissue: Strong staining was seen in most basal cells and some prickle cells (score = 2); (c) VSCC tissue: Strong staining was seen in most basal cells and some prickle cells (score = 2)

Immunohistochemical staining of CA-9

CA-9 expression was negative in control sections (Figure 2a), and was found only in 30% of VIN sections (Figure 2b). About 52% of VSCC sections with strong stained (score 1-2) (Figure 2c). Similarly, CA-9 expression also showed obvious difference between controls and VIN or VSCC sections (all P<0.05). However, no significant difference between VIN and VSCC was observed (P>0.05) (as shown in Table 1).

Immunohistochemical staining of VEGF

The staining pattern of VEGF was similar to GLUT-1. There was only 25% of control sections with weak (score 0-1) VEGF expression (Figure 3a). While strong staining (score 2–4) was found in about 60% of VIN sections

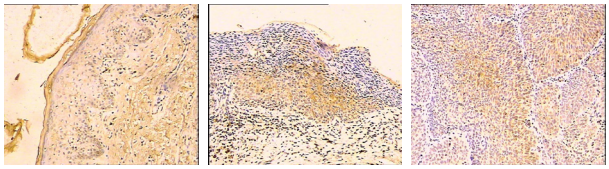

Figure 3. Immunohistochemical Staining of vascular endothelial growth factor (VEGF). (a) Control skin: Very weak staining sporadically expressed in basal cells and prickle cells (score = 0); (b) VIN tissue: Strong staining was seen in most basal cells and some prickle cells (score = 3); (c) VSCC tissue: Strong staining was seen in most basal cells and some prickle cells (score = 3)

(Figure 3b) and 25% of VSCC sections (Figure 3c). The distribution of percentage scores of VEGF showed obvious difference between controls and VIN or VSCC sections (all P<0.05). In addition, significant difference was also found between VIN and VSCC sections (P<0.05) (as shown in Table 1).

Correlations between GLUT-1, CA-9 and VEGF expression

In VIN and VSCC sections, the expression of GLUT-1 was found to correlate with VEGF expression (rs=0.743, P=0.014; rs=0.768, P<0.001; respectively). A significant positive correlation was found between CA-9 and GLUT-1 expression in VIN sections (rs=0.781, P=0.008), but no in VSCC sections (rs=0.271, P=0.19). However, there was no association between CA-9 and VEGF both in VIN and VSCC sections (rs=0.565 P=0.089; rs=0.150, P=0.475; respectively).

Discussion

Hypoxia is a consequence of early tumor cell proliferation on epithelial surfaces, which are separated from the underlying blood supply by the intact basement membrane (Fang et al., 2008). As tumor cells proliferate further away from the basement membrane, the diffusion-reaction kinetics of substrate and metabolite flow to and from the blood vessels result in regional hypoxia and acidosis. Molecules involved in the so-called hypoxic response of tumor cells have been considered endogenous hypoxia markers (Gillies and Gatenby, 2007). These proteins, including HIF-1α, GLUT-1, CA-9 and VEGF, can be detected by immunohistochemical staining in pathologic material and indirectly reflect the tumor oxygenation status and prognosis (Brown, 2000). Endogenous hypoxia markers have the potential to indicate therapeutically relevant levels of hypoxia within tumors and predict a benefit from specific hypoxia-directed treatment (Vordermark and Brown, 2003). Assessing hypoxia using endogenous molecular markers would be a cost effective approach that could be used by oncologists worldwide (Le et al., 2007). Endogenous molecular markers need not additional invasive procedure beyond that of a tumor biopsy at diagnosis (Davda and Bezabeh, 2006). However, single endogenous molecular markers can be influenced by factors other than hypoxia. Therefore, the inclusion of several endogenous markers could improve the specificity of such an approach to identifying patients with hypoxic tumors. To test this hypothesis, we investigated the expression of four endogenous molecular markers (HIF-1α, GLUT-1, CA-9 and VEGF) in VSCC by immunohistochemistry. Correlations between the expression of the different markers and clinical tumor and patient characteristics were studied.

It is known that pathophysiological phenomena, including glucose transport, glycolysis, angiogenesis, erythropoiesis and the inhibition of apoptosis, are usually regulated by HIF-1α. In this regulation, the expression level of the HIF-1α subunit is a determinant of HIF-1 transcription activity and the loss of the von Hippel-Lindau (VHL) tumor suppressor gene results in constitutive high-level expression of HIF-1α. Therefore, HIF-1α can adapt the cellular environment to a hypoxic status by inducing the expression of various hypoxia response molecules. It has been observed that increased HIF-1α is overexpressed as a result of intratumoral hypoxia, leading to treatment failure and a poor prognosis for the malignancies (Semenza, 2009). In this study, immunohistochemical analysis also revealed increased levels of HIF-1α protein in all healthy controls, VIN and VSCC sections. In general, when compared to non-malignant tumor cells or normal tissue cells the glucose utilization rate is increased in malignant tumor cells (Ortega et al., 2009). GLUT-1 are integral membrane glyco-proteins that play a key role in facilitating glucose transport (Airley et al., 2003). Various studies have shown a close relationship between GLUT-1 expression and carcinogenesis, tumor development or the unfavorable prognosis of various malignancies (Iida et al., 2008). Our study found that GLUT-1 expression was found in a small part of control sections, but in the majority of VIN and VSCC sections. In addition, we also found a significant difference between control and VIN or VSCC sections in GLUT-1 expression. These results also suggest that GLUT-1 is a useful marker for VSCC and should be included in the vulvar carcinomas immunophenotyping thus aiding in the correct diagnosis of these lesions. CA-9 is also one of the best characterized targets of HIF-1α, which is frequently overexpressed in human tumors and is associated with poor prognosis (Loncaster et al., 2001). Expression of CA-9 is regulated through both the HIF-1α and unfolded protein response hypoxia response pathways in vitro and in vivo (van den Beucken et al., 2009). In this study, we have also investigated the expression of CA-9 during hypoxia. We found that the expression of CA-9 was negative in healthy controls, but it was found in VIN and VSCC sections with strong staining. Similarly, the expression of CA-9 in VIN and VSCC were obviously higher that healthy controls. Our data indicate that CA-9 might be a surrogate marker of tumor hypoxia used to be a prognostic indicator for VSCC. Growth of tumors depend on a further induction of vascular development for adequate oxygen and nutrient supply (Hendriksen et al., 2009). Angiogenesis of tumours might develop as a result of environmental conditions, such as hypoxia (Danielsen and Rofstad, 2000). If the oxygen supply is insufficient, the resulting hypoxia stimulates angiogenesis through upregulation of HIF-1α and VEGF (Hendriksen et al., 2009). VEGF upregulation is associated with a poor response to treatment and poor prognosis (Saidi et al., 2008). In our study, we demonstrate that there was only

25% of control sections with weak VEGF expression, while strong staining was found in about 60% of VIN sections and 25% of VSCC sections. VEGF expression showed significant differences between controls and VIN or VSCC sections. Our results indicate that hypoxia can increase the angiogenic potential of VSCC tumor cells by increasing the secretion of VEGF. Transient hypoxia might promote the malignant progression of tumors by temporarily increasing the angiogenic potential of tumor cells. Taken together, these data support the hypothesis that the expression of endogenous hypoxia markers might be potential diagnostic and prognostic markers for VSCC.

In summary, this study indicates that expression of endogenous hypoxia markers (HIF-1α, GLUT-1, CA-9 and VEGF) might involved in the malignant progression of VSCC. The expression of GLUT-1, CA-9 and VEGF might be regulate by HIF-1α. Further, the expression of GLUT-1 was found to correlate with VEGF in VSCC tumor cells. There was no significant correlation between CA-9 and GLUT-1/VEGF expression in VSCC tumor cells. A limitation of this study is the relatively small sample size and the heterogeneity of the material. This precludes firm conclusions and verification of the results is needed in a larger patient cohort.

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