RESEARCH ARTICLE

CD44v3 and VEGF-C Expression and its Relationship with Lymph Node Metastasis in Squamous Cell Carcinomas of the Uterine Cervix

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Abstract

Background: To investigate the expression of CD44v3 and vascular endothelial growth factor-C (VEGF-C) and their relationship with lymph node metastasis in squamous cell carcinomas (SCC) of the uterine cervix. Materials and Methods: Expression of CD44v3 and VEGF-C was analyzed in 109 cases of cervical SCC by immunohistochemistry (IHC). The relationship was analyzed between expression and the patient age, histological differentiation, formation of tumor emboli in lymphoid vessels, lymph node metastasis, FIGO staging, and TNM classification. Results: Expression rates for both CD44v3 and VEGF-C were 43.1% in cervical SCC. The cells with positive immunohistochemical staining of CD44v3 were distributed mainly around the keratin pearls in well differentiated carcinomas, but distributed diffusely in the moderately and poorly differentiated lesions. VEGF-C was found stained positively in most of the tumor cells. There were differences in expression between normal epithelium and atypical hyperplasia as well as carcinoma. Both CD44v3 and VEGF-C were found to be associated positively with lymph node metastasis and TNM classification (both p=0.000). Neither CD44v3 nor VEGF-C was found to be associated with patient age, histological differentiation, formation of tumor emboli in lymphoid vessels and FIGO staging. CD44v3 was found to be associated with VEGF-C positively (p=0.000). Conclusions: Abnormal expression of CD44v3 and VEGF-C is associated closely with the lymph node metastasis in cervical SCC, and these agents may cooperate in carcinogenesis and development of metastatic lesions.

Keywords: Squamous cell carcinomas - uterine cervix - CD44v3 - vascular endothelial growth factor-C

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Introduction

Though the cancer death rate of cervical carcinoma decreased because of attractive improvements in early diagnosis and treatment over the past 20 years, cervical carcinoma continues to be one of the major causes of cancer-related deaths in women (Jemal et al., 2010). The most common histological type of cervical carcinoma is squamous cell carcinoma. Lymphatic spread is more typical of carcinomas, and that do affect seriously to the patients' prognosis. The trans-membrane receptor protein CD44 belongs to the family of cell-surface adhesion molecules mediating cell-cell and cell-matrix interactions and it has been shown that the expression of CD44 isoform, CD44v3, play an important role in the metastasis of human malignancies (Wang et al., 2007). The vascular endothelial growth factor-c (VEGF-C) which can promote intratumoral lymphangiogenesis is overexpressed in different malignancies and associated with lymph node metastasis and poor prognosis (Inoue et al., 2008; Duan et al., 2012; Wang et al., 2012; Liu et al., 2012). In this study, we examined the expression of CD44v3 and VEGF-C in 109 cases of cervical SCC immunohistochemically. And the relationship was analyzed between the expression and the patients' age, histological differentiation, formation of tumor embolus in lymphoid vessels, lymph node metastasis, FIGO staging, and TNM classification.

Materials and Methods

Patients and tissue samples

All evaluated samples of formalin-fixed, paraffin-embedded from patients of Chinese origin with cervical SCC for IHC were collected from the department of pathology in Sun Yat-sen Memorial Hospital from December 2004 to December 2008. They were sampled one from three according to their case numbers. All these cases have undergone radical hysterectomy and lymph node dissection. The median age at the time of diagnosis was 54 years (range 28 to 61), including 36 patients ≤ 40 years. None of the patients had received chemotherapy of radiation before surgery. The tumors were graded

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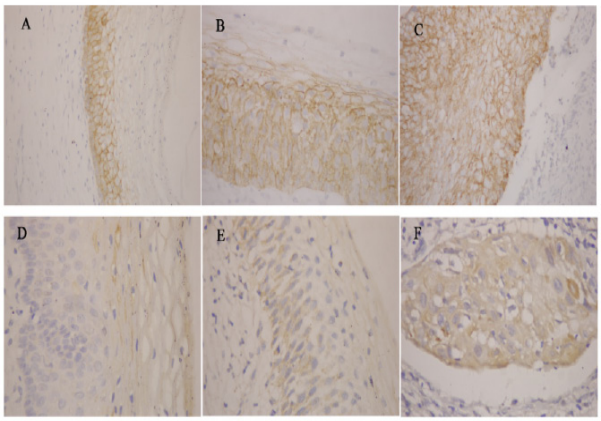

Figure 1. Expression on Immunohistochemistry in Squamous Cell Carcinomas of the Uterine Cervix. (A) CD44v3 located in the basal and basolateral layer cells of normal epithelium adjacent to carcinoma. (B) CD44v3 located in the lower second thirds cells of metaplasia epithelium adjacent to carcinoma. (C) CD44v3 expressed diffusely in the poorly-differentiated squamous carcinoma. (D) VEGF-C expressed randomly in the normal epithelium adjacent to carcinoma. (E) VEGF-C located in the lower half cells of metaplasia epithelium adjacent to carcinoma. (F) VEGF-C expressed diffusely in the moderately-differentiated squamous carcinoma. S-P, original magnification×200

histologically as well-differentiated (30 patients, 27.6%), moderately differentiated (48 patients, 44.0%), and poorly differentiated (31 patients, 28.4%). Of the 109 cervical SCC patients, local lymph node metastasis occurred in 26 cases (23.9%) and the formation of tumor embolus in lymphoid vessels in lymphoid vessels was found in 24 cases (22.2%) without lymph node metastasis. According to the FIGO system of cervical carcinomas (2004), tumors were classified as stage IA2 (2 patients, 1.8%) IB1 (2 patients, 1.8%) IB2 (62 patients, 56.9%) IIA (28 patients, 25.7%) IIB (15 patients, 13.8%), and according to the WHO classification of cervical carcinomas (Tavassoli FA, Devilee P et al., 2003) as stage I(53 patients, 48.6%), stage II (30 patients, 27.5%), and stage III (26 patients, 23.9%).

Immunohistochemistry

Protein expression of CD44v3 and VEGF-C was performed on cervical SCC cervical SCC tissues fixed by 10% neutral formalin and embedded by paraffin. Slides of 4 μm sections were deparaffinized with xylene and antigen retrieval was accomplished by using microwave oven. The sections were then incubated in 3% hydrogen peroxide at room temperature for 15 min to block endogenous peroxidase activity. Slides were then incubated with monoclonal anti-CD44v3 (clone 3G5) and polyclonal anti-VEGF-C (Zhongshan-Golden Bridge Biological Technology Co., Beijing) at 1:50 dilutions at 4°C overnight. After warming up at room temperature for 30 minutes, the slides were rinsed three times in PBS for 5 min each and then incubated with the secondary biotinylated antibody and streptavidin-HRP conjugate complex for 30 and 45 minutes at 37°C, respectively. After washing three times with PBS, the staining was performed using 3, 3'-diaminobenzidine. Sections were counterstained with hematoxylin. The sections of tongue carcinoma tissues known for the antibody stained positive were used as positive controls, and normal goat serum and PBS substituting the primary antibody were used as negative controls.

Evaluation of immunostaining

The judgement whether the tumor tissues were positive or not was performed by two pathologists (YQ L and HG L). Staining was considered positive when the cell membrane (for CD44v3) or cytoplasmic (for VEGF-C) staining was observed in at least 10% of the neoplastic cells. In nine cases, discordant results were obtained. These slides were reevaluated together and a consensus was reached.

Statistical analysis

Data were analyzed with SPSS for Windows (Version 8.0) by using the Mann-Whitney U test, Kruskal-Wallis Test and Pearson's method. P values <0.05 were considered statistically significant.

Results

Expression of CD44v3 and VEGF-C in 109 carcinoma tissues of cervical SCC

Both expressive rates of CD44v3 and VEGF-C were 43.1% (47 out of 109) in cervical SCC (Figure 1C and F). Most of the cells with positive staining of CD44v3 in well differentiated carcinomas distributed around the keratin pearl, but distributed diffusely in the mediate and poorly differentiated. Meanwhile, CD44v3 immunoreactivity was found in the basal and basolateral layer cells of epithelium in the squamous epithelium adjacent to carcinoma, which was similar to the distribution in well differentiated carcinoma. In the normal squamous epitheliums beside carcinoma, CD44v3 expression was limited to the basal one third cells while positive in the lower two thirds cells of the atypical hyperplasia beside carcinoma excepted parakeratosis. VEGF-C was found stained positively in most of the tumor cells, and it expressed randomly in the normal epithelium adjacent to carcinoma while was found in the lower half cells of metaplasia epithelium adjacent to carcinoma (Figure 1A, B, C, D, E and F).

Correlation of CD44v3 expression with clinicopathological parameters of cervical SCC

There were 23 cases positive for CD44v3 out of 26 cases (88.4%) of cervical SCC with lymph node metastasis, while 24 out of 83 cases (28.8%) of cervical SCC without lymph node metastasis. The positive rate of the group with lymph node metastasis was significantly higher than that of group without metastasis (Z=-5.325, p=0.000, r=0.512). The positive rate of CD44v3 was 88.4% (23/26) in stage III (TNM), which was significantly higher than that in stage II(TNM) of 20.0% (6/30), and stage I(TNM) of 33.9% (18/53). A linear trend in CD44v3 expression level with increasing TNM stage was observed (Z=-3.526, p=0.000, r=0.379) (Table 1). In 62 cases of FIGO IB2, all the 12 cases with lymphoid node metastasis were positive for CD44v3, and 16 were positive out of 50

Table 1. The Expression of CD44v3 and VEGF-C in 109 Cases of Cervical SCC

Parameters	n	CD44v3		VEGF-C	
		-	+	-	+
Age					
≤40	36	24	12	23	13
>40	73	38	35	39	34
Differentiation					
Well	30	18	12	20	10
Mediate	41	24	17	21	20
Low	38	20	18	21	17
Lymphoid metastasis					
No	83	59	24*	56	27*
Yes	26	3	23	6	20
Embolus in lymphoid vessels					
No	85	47	38	49	36
Yes	24	15	9	13	11
TNM stage					
I	53	35	18*	36	17*
II	30	24	6	20	10
III	26	3	23	6	20
FIGO stage					
IA2	2	0	2	1	1
IB1	2	1	1	1	1
IB2	62	34	28	38	24
IIA	28	19	9	15	13
IIBI	15	8	7	7	8

*p<0.05

Table 2. Relationship of CD44v3 with VEGF-C Cervical SCC

		VEGF-C	
		-	+
CD44v3	-	46	16*
	+	16	31

P=0.000

cases without lymphoid node metastasis. The positive rate of the group with lymph node metastasis was significantly higher than that of group without metastasis too (Z=-4.216, p=0.000, r=0.540).

Among 24 cases with the formation of tumor embolus in lymphoid vessels, 9 cases (37.5%) were found positive for CD44v3, among 85 cases of non embolus, 38 cases (44.7%) were found positive. No significance was found between the two groups with or without tumor embolus tumor embolus (Z=-1.094, p=0.274). Meanwhile, among the 83 cases without lymphoid node metastasis, 22 cases were found tumor embolus in lymphoid vessels. 7 cases (31.8%) were found positive for CD44v3 among those 22 cases with tumor embolus in lymphoid vessels, and 18 (29.5%) found positive among the other 61 cases without tumor embolus in lymphoid vessels. No significance was found between the two groups without lymphoid node metastasis either (Z=-0.201, p=0.841). No relationship was found between the expression of CD44v3 and the age, differentiation and FIGO stage (P>0.05) (Table 1).

VEGF-C expression correlation with clinicopathological parameters of cervical SCC

There were 20 cases positive for VEGF-C out of 26 cases (76.9%) of cervical SCC with lymph node metastasis, while 27 out of 83 cases (32.5%) of cervical SCC without lymph node metastasis. The positive rate of the group with lymph note metastasis was significantly higher than that of group without metastasis (Z=-3.970, r=0.382, p=0.000). The positive rate of VEGF-C was 76.9% (20/26) in stage III (TNM), which was significantly higher than that in stage II(TNM) of 33.3% (10/30) and stage I(TNM) of 32.0% (17/53). A linear trend in VEGF-C expression level with increasing tumor stage was observed (Z=-3.244, p=0.001, r=0.333) (Table 1). In 62 cases of FIGO IB2, 8 out of the 12 cases with lymphoid node metastasis were positive for VEGF-C, and 16 were positive out of 50 cases without lymphoid node metastasis. The positive rate of the group with lymph node metastasis was significantly higher than that of group without metastasis too (Z=-2.196, p=0.028, r=0.281).

Among 24 cases with the formation of tumor embolus in lymphoid vessels, 11 cases (45.8%) were found positive for VEGF-C, among 85 cases of non embolus, 36 cases (42.4%) were found positive. No significance was found between the two groups with or without lymph node metastasis (Z=-0.303, p=0.762). Meanwhile, among the 83 cases without lymphoid node metastasis, 22 cases were found tumor embolus in lymphoid vessels. 11 cases (50.0%) were found positive for VEGF-C among those 22 cases with tumor embolus in lymphoid vessels, and 17 (27.9%) found positive among the other 61 cases without tumor embolus in lymphoid vessels. No significance was found between the two groups without lymphoid node metastasis either (Z=-1.871, p=0.061). No relationship was found between the expression of VEGF-C and the age, differentiation and FIGO stage (P>0.05) (Table 1).

Relationship between CD44v3 and VEGF-C expression in carcinoma tissues of cervical SCC

In 109 carcinoma tissues of cervical SCC, 31 were found to be positive for both CD44v3 and VEGF-C, and 46 negative for both. The expression of CD44v3 was found correlated positively with the expression of VEGF-C (Z=-4.173, r=0.402, p=0.000) (Table 2). In 62 cases of FIGO IB2, 18 were found to be positive for both CD44v3 and VEGF-C, 10 positive for CD44v3 and 6 positive for VEGF-C only, and 28 negative for both. The expression of CD44v3 was found correlated positively with the expression of VEGF-C (Z=-3.722, r=0.477, p=0.000).

Discussion

The overall 5-year survival rate for squamous cell carcinomas of the uterine cervix, one of the most common cancers in women all over the world, is still low, largely due to the dissemination of cancers via the lymphatic pathway. Lymph node involvement is a key feature and an independent prognostic factor of cervical squamous cell carcinoma. Studies suggest that CD44v3 and VEGF-C correlates with lymph node metastasis (Wang et al., 2007; Duan et al., 2012; Xie et al., 2013).

The trans-membrane receptor protein CD44 belongs to the family of cell-surface adhesion molecules mediating cell-cell and cell-matrix interactions (Jalkanen et al., 1991). CD44 proteins are encoded by a single gene comprised of

20 exons located on chromosome 11. By modification of pre-messenger RNA, i.e., alternative splicing, numerous isoforms of the CD44 protein are produced (CD44 isoforms CD44v1-CD44v10) (Matsumura et al., 1992; Screaton et al., 1992). And the expression of some special CD44 isoforms has been shown to be associated with metastasis and poor prognosis (Tawfik et al., 2007; Naor et al., 2008; Banky et al., 2012; Kunlathida et al., 2012). The variant exon v3 may be modified by heparan sulfate and confers the capacity to bind heparin-binding growth factors *in vitro* (K L et al., 1995). This may increase the local concentration of growth factors at the cell surface and further promote activation of high-affinity growth factor receptors (Schlessinger et al., 1995). In this study, we found that immunostaining for CD44v3 was positive in the atypical hyperplasia of squamous epitheliums beside carcinoma, which was similar to the CD44v3 expression in a premalignant lesion of oral epithelium (Li et al., 2009) and the esophagus the esophagus (Roye et al., 1996). The distribution of cells with CD44v3 expression in the squamous epithelium adjacent to carcinoma was different from that in tissues of cervical SCC, and similar expression was found in well differentiated carcinomas distributing in the basal and basolateral layer cells around the keratin pearl mainly. However, the cells with CD44v3 positive staining distributed diffusely in the mediated and low differentiated ones, which prompted that cells with stronger proliferative ability might express more CD44v3, cells with weaker proliferative ability or without it, such as the cells in the center of keratin pearl, would not express CD44v3. It has been suggested that CD44v3-HSPG(heparin-binding growth factors) expression may promote the metastatic phenotype through the sequestration and presentation of heparin-binding growth factors (van et al., 1999) . CD44v3 expression is up-regulated in a variety of human tumors and their metastatic lesions (Fox et al., 1993; Gotley et al., 1996). A previous study has confirmed the CD44v3 expression is an independent prognosticator in early-stage cervical carcinoma (FIGO IB)(Speiser et al., 1997). This study showed that the positive rate of CD44v3 in the group with lymph node metastasis was significantly higher than that without metastasis both in the total samples and the 62 cases of IB2, and the positive rate in stage III was significantly higher than in stage II and I. The results demonstrated that CD44v3 might play an important role in the metastasis of squamous cell carcinomas of the uterine cervix.

VEGF-C is overexpressed in many human malignancies and plays a crucial role in tumor lymphangiogenesis, which induces the formation of additional lymphatic vessels and provides routes by which tumors cells metastasize to distant sites. Activation of the VEGF-C/VEGFR-3 axis in lymphatic endothelial cells can facilitate metastasis by increasing the formation of lymphatic vessels within and around tumors (Su et al., 2007). In our study, VEGF-C expressed randomly in the normal epithelium adjacent to carcinoma while was found in the lower half of metaplasia epithelium adjacent to carcinoma and in most of the tumor cells. It has been suggested that VEGF-C expression is a diagnostic marker for early stages of cervical carcinoma, based on the fact that the expression level of VEGF-C whose upregulation is closely related to the presence of high risk human papillomavirus (HR-HPV) was weak in low grade CIN and strong in CIN III and cancer lesions, but it has no relationship with prognosis whatever by single factor analysis or multiple-factor analysis in cervical carcinoma (Branca et al., 2006). And the VEGF-C up-regulation in serum appeared to be a unique marker for an early diagnosis of cervical carcinoma metastasis, measuring serum VEGF-C levels would lead to an early non-invasive and specific diagnosis of potential metastasis in women with cervical carcinoma (Mathur et al., 2005). Meanwhile some researchers suggested a potential role for VEGF-C in tumor-induced lymphangiogenesis represented by high peritumoral lymphatic vessel density (LVD) in early-stage of cervical SCC, which might be one of the mechanisms leading to lymphatic invasion and metastatic spread (Gombo et al., 2005). The expressions of VEGF-C in 109 cervical squamous cell carcinomas were assessed in relation to clinicopathological features of tumors such as the patients' age, histological differentiation, formation of tumor embolus in lymphoid vessels, lymph node metastasis, FIGO staging, and TNM classification. In this study, we found that the distribution of cells with VEGF-C expression was diffusive, and VEGF-C was associated positively with lymph node metastasis both in the total samples and the 62 cases of IB2 (FIGO), and associated positively with TNM stage. Majority of cervical carcinomas metastasize in lymph nodes, which correlate with the prognosis of the patients. Many studies had provided evidence supporting the association of VEGF-C expression with interstitial infiltrates, pelvic lymph node metastasis and lymphatic invasion in cervical carcinoma (Hashimoto et al., 2001). The close relationship between VEGF-C expression and lymph node metastasis could be explained by a hypothesis that overexpression of VEGF-C in carcinoma stimulates lymphangiogenesis and enhances the invasion of cancer cells, and leads to lymph node metastasis.

Interestingly, neither CD44v3 nor VEGF-C was found to be correlated with the formation of tumor embolus in lymphoid vessels of the whole cases and the cases without lymphoid metastasis. And 2 cases were found the formation of tumor embolus in lymphoid vessels in the 26 cases with lymphoid node metastasis Data not shown), which suggests that most of the tumor cells in the lymphoid vessels failed to develop metastasis. That means the inefficiency of metastasis might occur in cervical SCC usually.

In the normal squamous epitheliums beside carcinoma, CD44v3 expression was limited to the basal one third cells while positive in the lower two thirds cells of the atypical hyperplasia beside carcinoma excepted parakeratosis, and so do VEGF-C. This reminds us abnormal expression of CD44v3 and VEGF-C may be detected in early-stage SCC of the uterine cervix, and participates in the process of carcinogenesis.

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