RESEARCH ARTICLE

Prognostic Significance of α5β1-integrin Expression in Cervical Cancer

Hua-Yi Wang[1,2], Zhe Chen[3], Zhu-Hui Wang[3], Hong Wang[2], Li-Ming Huang[1]*

Abstract

The purpose of this study was to evaluate the association of expression of α5β1-integrin with clinicopathologic features and prognosis in cervical cancer. Levels of α5β1-integrin in normal cervical mucosa and cervical cancer tissue were detected with immunohistochemistry. Survival analysis by the Kaplan-Meier method was performed to assess prognostic significance. α5β1-integrin expression was detected in 84.6% (143/169) cervical cancer samples, significantly different from that in normal cervical mucosa ($P < 0.05$). Positive expression rates of α5β1-integrin in patients with poor histologic differentiation, lymph node metastasis, and recurrence were elevated. Using Kaplan–Meier analysis, a comparison of survival curves of low versus high expression of α5β1-integrin revealed a highly significant difference in human cervical cancer cases ($P < 0.05$), suggesting that overexpression of α5β1-integrin is associated with a worse prognosis.The α5β1-integrin promotes angiogenesis and associates with lymph node metastasis, vascular invasion and poor prognosis of cervical cancer. The current study indicated that α5β1-integrin may be an independent prognostic factor for cervical cancer patients.

Keywords: Cervical cancer - α5β1-integrin - prognosis - survival

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Introduction

Cervical cancer (CC) is the second most common malignant diseases of women in the world. More than 500,000 new cases of cervical cancer were reported each year worldwide, of which approximately 1/3 from China mainland. So, cervical cancer is a cause of significant morbidity and cancer-related mortality in China women. With the improvement of modern irradiation techniques and development of novel drugs, cervical cancer remains an unsolved problem of oncology both due to the increased rate of local failures and of the distant metastasis. Although the death rate for cervical cancer has decreased dramatically since the introduction of cervical cytology as a widespread screening procedure, the survival rate at advanced stages of disease has not improved. Therefore, characterization of identifiable molecular markers may play an important role in understanding of molecular pathogenesis and in identifying latently prognostic biomarkers for cervical cancer.

Numerous studies demonstrated that α5β1-integrin expression levels are altered in many types of cancer (Morozevich et al., 2009), there may be involved in modulating effect on several signalling pathways ,which are closely related to the regulation of cell survival, proliferation ,differentiation and apoptosis, and in stimulating tumor angiogenesis (Zeng et al., 2009) Although it has been shown that α5β1-integrin is a poor prognostic factor in human breast cancer and chondrosarcoma (Loredana et al., 1999; Tang et al., 2012), the clinicopathological and prognostic significance of α5β1-integrin have not yet been elucidated in cervical cancer.

Herein, in the present study, we compared the expression of α5β1-integrin in normal human cervical tissue and in cervical cancer tissue. We also investigated the associations between α5β1-integrin and clinicopathological features and prognosis. We found a correlation between α5β1-integrin overexpression and poor survival, which suggests that α5β1-integrin may be an independent prognostic factor for cervical cancer patients.

Materials and Methods

Patients and tissue samples

Cervical cancer tissue were obtained from 169 patients in the Department of Obstetrics and Gynecology, Jingzhou Second People Hospital and Zhongnan Hospital of Wuhan University, between May 2002 and May 2006. Clinicopathological characteristics of patients are summarized in Table 1. The study protocol was approved by Ethics Committee of the two hospitals and all participants signed an informed consent form. No patient had received radiotherapy, chemotherapy, or other treatment prior to surgery. Following surgical removal, the tissue sample was formalin-fixed and paraffin-embedded

*[1]Department of Oncology, Zhongnan Hospital of Wuhan University, Wuhan, [2]Department of Pathology, [3]Department of Obstetrics and Gynecology, Jingzhou Second People Hospital, Jingzhou, China *For correspondence: xiexianmu2008@126.com*

for histopathologic diagnosis and immunohistochemical examination. The 68 normal cervical tissues from patients who underwent hysterectomy for reasons other than neoplasia of either the cervix or the endometrium were used as a control group. During the follow-up period from the date of surgery until May 31, 2012, 29 patients died and 128 were alive (median follow-up time, 54.9 mo, range, 3-82 mo).

Immunohistochemistry analysis

The paraffin embedded cervical cancer tissues and distal normal mucosa tissues were cut at 4 μm and mounted on glass slides. Then the slides were dewaxed in xylene and rehydrated in ethanol, and treated with a solution of peroxidase-blocking reagent (Dako, Glostrup, Denmark) to exhaust endogenous peroxidase activity. They were put in 0.01 mol/L citrate buffer at pH 6.0 for 15 minutes in an 800W microwave oven and then left at room temperature for 20 minutes to expose antigen hidden inside the tissue due to formalin fixation. To inhibit non-specific antigen-antibody reactions possible in immunohistochemical staining, protein blocker (Research Genetics, Huntsville, AL, USA) was used for 5 minutes and the slides were washed thoroughly with PBS buffer. Then the slides were incubated overnight with the primary antibodies against α5β1-integrin (1:100; mouse polyclonal antibody, ECM410, Tianyuan Huida Bio-engineering Limited Company, Wuhan, China) at 4 centigrade. Biotinylated goat anti-rabbit secondary antibody (1:200; BA1003, Boster Bio-engineering Limited Company, Wuhan, China) was applied for 20 minutes at room temperature, followed by further washing with buffer to remove unbound antibody. A complex of avidin with horseradish peroxidase was then applied for 20 minutes at room temperature. For color development, the slides were stained with 3,3'diaminobenzidine (DAB, Sigma-Aldrich, St Louis, MO, USA) then were counterstained with hematoxylin. A reddish brown precipitate in the cytoplasm of cells indicated a positive reaction. In each immunohistochemistry run, positive sections provided by reagent company served as positive controls and omission of the primary antibody served as negative control.

Quantification of IHC staining

Assessment of the staining was scored independently by two investigators (Huayi Wang and Liming Huang) who were blinded to all clinical data. The allocation of tumors and scoring staining by the two investigators was similar. In cases of disagreement, slides were reevaluated and discussed until a consensus was achieved. α5β1-integrin staining was considered positive if there was cytoplasm expression. Staining was graded (0, negative; 1, weak; 2, moderate; 3, strong) and percentage of positive staining cells was counted (0, < 10%; 1, 11%-50%; 2, 51%-75%; 3, > 76%). The final score was determined by the combined staining score and proportion score (intensity score*proportion score). The total score ranged from 0 to 9. The immunoreactivity was divided into 3 levels on the basis of the final score: negative immunoreactivity was defined as a total score of 0; low immunoreactivity, as a total score of 1 to 4; and high immunoreactivity, as a total score higher than 4.The final results were subjected to statistical analysis.

Statistical Analysis

Associations among categorical variables were assessed using Fisher's exact probability test or the x^2 test. Overall survival was measured by the Kaplan-Meier method. The prognostic value of the 6 variables was tested by univariate analysis using the log-rank test. Multivariate Cox proportional hazard models were used to define the potential prognostic significance of individual parameter. A *P*-value of less than 0.05 was considered significant. All statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Pattern of α5β1-integrin expression in cervical cancer and normal mucosa

The α5β1-integrin staining was as performed in 169 cervical cancer patients and 68 cases of normal tissues by immunohistochemistry. The α5β1-integrin expression was detected in 84.6% (143/169) cervical cancer, and in 33.8% (23/68) distal normal mucosa (Figure 1). The expression of α5β1-integrin was found in cytoplasm only. The difference of α5β1-integrin expression between cervical cancer and normal mucosa was statistically significant (χ^2 = 59.616, $P < 0.001$).

Correlation of α5β1-integrin expression and clinicopathological features in cervical cancer

When comparing the α5β1-integrin status with

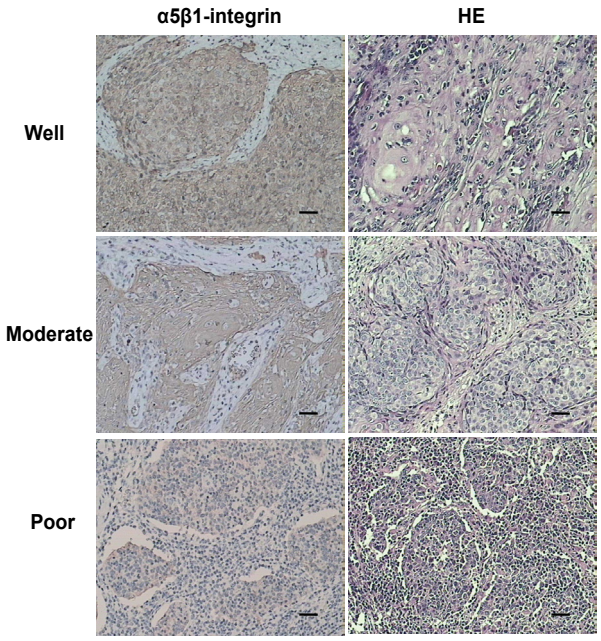

Figure 1. Expression of α5β1-integrin by Immunohistochemistry at the Invasive Front of Cervical Cancer Tissue with Well-(upper panel), Moderately (middle panel), and Poorly (lower panel) Differentiated Cervical Cancer. The right panels show the sections stained with H&E. Bar indicates 20 μm. The scores for α5β1-integrin in tissue with well, moderate, and poor differentiation were 0, 3, and 9, respectively

Table 1. Relationship Between α5β1-integrin Expression Levels of Tumors and Clinicopathological Feature

Variable	Cases	α5β1-integrin			
		N	L	H	*P*
Age					
<40years	67	11	44	12	0.513
≥40years	102	15	61	26	
Tumor size					
<4cm	77	12	48	17	0.992
≥4cm	92	14	57	21	
Histologic type					
Squamous carcinoma	121	18	82	21	0.112
Adenocarcinoma	27	5	13	9	
Adenosquamous carcinoma	21	3	10	8	
Histologic differentiation					
Well	56	13	35	8	0.005**
Moderate	46	11	24	11	
Poor and Unknown	67	2	46	19	
FIGO stage					
I	31	5	20	6	0.165
II	102	12	69	21	
III	30	7	15	8	
IV	6	2	1	3	
Metastasis of lymph node					
N0	95	9	84	2	0.000**
N (+)	74	17	21	36	
Follow-up					
Live without recurrence	128	22	90	16	0.000**
Died of recurrence	29	3	7	19	
Live with recurrence	12	1	8	3	

N, negative; L, low expression; H, high expression; Statistically significant *$P < 0.05$, ** $p < 0.01$

Table 2. Univariate Cox Regression Analysis of Overall Survival

Factor	Log-rank test	*P*
Age (<40, ≥40 y)	0.001	0.998
Histologic differentiation (well, moderate, poor and unknow)	30.711	0.000**
FIGO Stage (I, II, III, IV)	1.899	0.594
Nodal metastasis (N0, N(+))	9.523	0.002**
Recurrence	86.98	0.000**
α5β1-integrin expression (negative, low, high)	33.951	0.000**

Statistically significant *$P < 0.05$, **$p < 0.01$; N0, no nodal metastasis; N(+), nodal metastasis; CI, confidence interval

clinicopathological variables, we found significant positive correlations between α5β1-integrin expression and histologic differentiation ($P = 0.005$), lymph node metastasis ($P = 0.000$), and recurrence ($P = 0.000$) (Table 1).

Relationship between α5β1-integrin and overall survival of cervical cancer patients

At the time of the last follow-up, 140 (82.8%) of 169 patients were alive and disease-free, 12 (7.1%) were alive with recurrent disease, and 29 (17.2%) died of recurrent

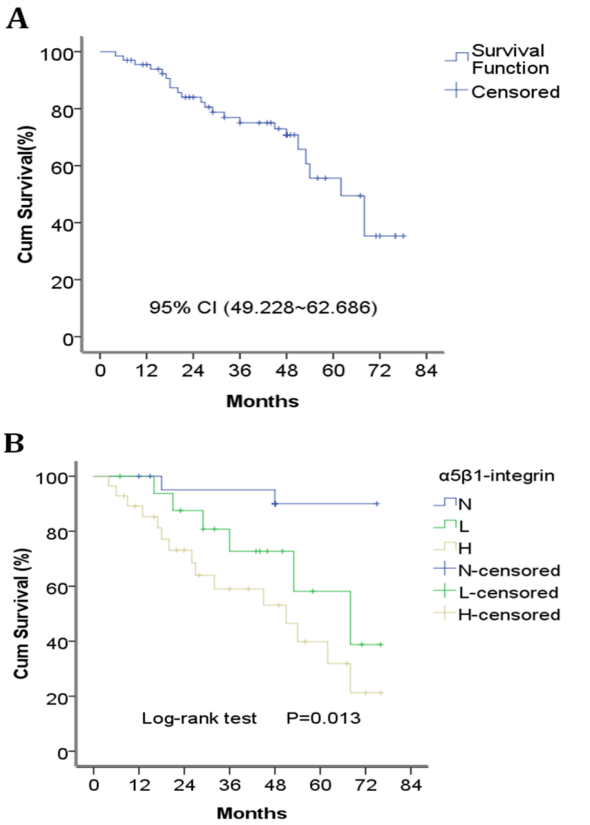

Figure 2. Survival Curves of 169 Patients with Cervical Cancer. A: Overall survival curve; B: Survival of patients with tumors lacking (N) or expressing low (L) or high (H) levels of α5β1-integrin (log-rank test, $P = 0.013$)

tumor. The overall survival curve for the 169 cases is shown in Figure 2 A. In the univariate Cox proportional hazard regression model analysis shown in Table 2, histologic differentiation ($P < 0.001$), node status ($P = 0.002$), recurrence ($P < 0$ .001), and expression intensity of α5β1-integrin ($P < 0.001$; Figure 2B) were significantly associated with overall survival. Consequently, patients with tumors with negative or low expression of α5β1-integrin had a better prognosis than those with tumors having high α5β1-integrin expression.

Discussion

Integrins are the principle adhesion receptors used by endothelial cells to interact with their extracellular microenvironment, they play important roles in the adhesion, migration, proliferation, survival and differentiation of the cells that form the vasculature (Hynes et al., 1999; Serini et al., 2002; Hynes et al., 2006; Hynes et al., 2007). Alterations in the repertoire and activity of integrins, as well as the availability and structural property of their ligands, regulate the vascular cell during the

Table 3. Multivariate Cox Regression Analysis of Overall Survival

Factor	*P*	Risk ratio	95% CI
Histologic differentiation (well, moderate, poor and unknow)	0.000**	2.261	1.637-3.124
Nodal metastasis (N0, N(+))	0.041*	1.614	1.019-2.556
Recurrence	0.000**	18.365	8.822-38.229
α5β1-integrin expression (negative, low, high)	0.001**	1.792	1.271-2.528

Statistically significant *$P < 0.05$, **$p < 0.01$; N0, no nodal metastasis; N(+), nodal metastasis; CI, confidence interval

growth or repair of blood vessel (Stupack et al., 2004). The altered integrins can change affinity and avidity for their extracellular matrix (ECM), and cancer cells become more adhesive and invasive, and lead to increased metastatic potential and enhanced angiogenic potential (Hood et al., 2002). In previous study, robust associations between altered α5β1-integrin expression and highly metastatic potential was revealed in human lung adenocarcinoma cell line (Takenaka et al., 2000). In the current study, we found that α5β1-integrin expression in cervical cancer tissues (143/169, 84.6%) remarkably higher than the corresponding normal mucosa (23/68, 33.8%), and there was statistically significant difference ($\chi^2 = 59.616$, $P < 0.001$). Our data implied that α5β1-integrin was closely correlated to cervical cancer cells biological events of tumor occurrence, invasion and metastasis.

Moreover, the overall survival of patients with high α5β1-integrin expression was significantly worse than that of patients with low or no expression. The univariate survival analysis revealed α5β1-integrin expression was a significant prognostic factor as well as histologic differentiation, node status, and recurrence. The status of α5β1-integrin expression might be dependent on the status of lymph node metastasis or other variables. So the multivariate Cox regression analysis for overall survival was performed, and multivariate analysis revealed that α5β1-integrin expression was picked up for its independent level of prognostic significance. The α5β1-integrin expression level plays one of the key roles in the biology of CC and defines a more aggressive tumor phenotype of CC. Preoperative adjuvant therapy in CC is designed to improve survival and reduce local recurrence. Our results also showed that the tumors with a strong expression of α5β1-integrin were associated with an increased recurrence, which suggests that patients with strong α5β1-integrin expression may be prone to metastasis. Therefore, α5β1-integrin expression was closely related to poor prognosis, and α5β1-integrin may serve as a marker for poor prognosis.

There are several possible mechanisms responsible for this association. Firstly, α5β1-integrin may be involve in promoting tumor angiogenesis (Caiado et al., 2012; Li et al., 2012). It is well known that the growth and spread of neoplasms depends on the establishment of an adequate blood supply (Fidler et al., 2000). Angiogenesis depends on endothelial cell interactions with the extracellular matrix (Eliceiri et al., 2001). The α5β1-integrin and its ligand, fibronectin, are clearly proangiogenic (Hynes et al., 2002). Global deletion of the α5 integrin gene results in an embryonic lethal phenotype, with aberrant blood vessel formation in the embryo (Yang et al., 1993). Similar vascular defects are also apparent in α5 integrin-null embryoid bodies and teratoma cells (Taverna et al., 2001). Recent research report that α5β1-integrin plays an important role in stimulating endothelial cell proliferation at an early step in the angiogenic process (Li et al., 2012). Secondly, α5β1-integrin may partake in activating MMP-2 (Bernard et al., 2006; Morozevich et al., 2009; Shi et al., 2011; Kesanakurti et al., 2012; Zhu et al., 2013). Invasion and metastasis of cancer have a close relationship with basement membrane adhesion and extracellular matrix degradation. Recently, accumulating evidence show α5β1-integrin mediated modulation of MMP-2 activity and suggest a direct interaction between MMP-2 and α5 integrin in melanoma and breast cancer cells (Mitra et al., 2003; Morozevich et al., 2008; Morozevich et al., 2009). Migrating astrocytes show co-localization of MMP-2 with β1 integrin at the cell periphery, indicating its significance in pericellular proteolysis (Ogier et al., 2006). Galina Morozevich's study showed that α5β1-integrin controls invasion of the breast cancer cells via regulation of MMP-2 collagenase expression which can occur either through signaling pathways involving PI-3K, Akt and Erk protein kinases and the c-Jun or via direct recruitment of MMP-2 to the cell surface (Morozevich et al., 2009). Thirdly, α5β1-integrin is also implicated to trigger Ras, MAP kinase, focal adhesion kinase (FAK), Src, Rac/Rho/cdc42 GTPases, PKC and PI3K (phosphatidylinositol 3-kinase) signaling pathways (Miyamoto et al., 1996; Aplin et al., 1998; Schwartz et al., 2000; Ridley et al., 2000). Integrins are widely recognized as important molecules for the transduction of positional cues from the ECM to the intracellular signaling machinery. Many of the signaling pathways and effectors activated by integrin ligation are also activated following growth-factor stimulation. The integrin and growth-factor mediated cellular responses may synergize and coordinate biochemical responses. The coordinated response of these inputs may direct the processes of cell migration, proliferation and differentiation.

Further study of the basic cell biological mechanisms of integrin-mediated cell adhesion and survival during angiogenesis will continue to provide insight into how to target tumor associated vasculature during angiogenesis to block tumor growth and metastasis and to prevent other diseases (Eliceiri et al., 2001). Thus, the utility of the expression of α5β1-integrin could open up a new window for the molecular marker and the treatment of CC.

In combination, our present study has underlineed the importance of α5β1-integrin in tumor initiation, progression and metastasis process,and the possible rationale underlying the relationship between α5β1-integrin and angiogenesis. Although further work is required to define the molecular mechanisms, the expression of α5β1-integrin may serve as a valuable tool of clinical assessment of tumour biological behaviour and prognosis in patients with cervical cancer.

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