Overexpression of fibulin-4 is associated with tumor progression and poor prognosis in patients with cervical carcinoma

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Abstract. Fibulin-4, a member of the fibulin family of extracellular glycoproteins, is implicated in the progression of a number of types of cancer. However, the function of fibulin-4 in cervical cancer progression remains unexplored. Fibulin-4 mRNA and protein expression levels in normal cervical tissue, cervical intraepithelial neoplasia (CIN), cervical carcinoma, highly invasive subclones and low-invasive subclones were evaluated by real-time reverse transcriptase-polymerase chain reaction and immunohistochemistry. Serum fibulin-4 levels in patients with CIN and cervical carcinoma were measured by enzyme-linked immunosorbent assay. To assess the angiogenic properties of fibulin-4, vascular endothelial growth factor (VEGF) expression and tumor microvessel density (MVD) were analyzed in the cervical carcinoma cases by immunohistochemistry. Fibulin-4 expression was upregulated in the cervical carcinoma cases, and was positively correlated with MVD and VEGF expression. Fibulin-4 overexpression and high serum levels were significantly associated with advanced stage, low differentiation, lymph node metastasis, and poor prognosis in patients with cervical cancer. Fibulin-4 expression was also found to be overexpressed in highly invasive subclones when compared with the low-invasive subclones. Fibulin-4 is a newly identified glycoprotein that is overexpressed in cervical carcinoma. Fibulin-4 promotes angiogenesis and is associated with poor prognostic clinicopathologic features. This study demonstrated that fibulin-4 may serve as a new prognostic factor and as a potential therapeutic target for patients with cervical carcinoma.

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Introduction

In women, cervical cancer is the second most common cancer in developing regions with more than 85% of the global burden occurring in developing countries (1). Although cervical cancer screening has reduced its incidence, ~30% of patients are still diagnosed at an advanced stage and ultimately show recurrence and metastasis (2). Therefore, investigation of the mechanisms of tumor invasion and metastasis will provide further insight into the occurrence and development of cervical cancer. In recent years, many genes such as secreted protein acidic and rich in cysteine (3), metastasis-associated 1 (4), and twist homolog 2 (5) have been found to be correlated with the progression of cervical cancer. However, few studies have explored the relationship between fibulin-4 and cervical cancer progression and prognosis.

Fibulin-4, also known as endothelial growth factor (EGF)-containing fibulin-like extracellular matrix protein 2 (EFEMP2), mutant p53 binding protein 1 (MBP1) or UPH1, is a 443-amino acid secreted protein that contains six EGF-like calcium-binding domains and belongs to the fibulin family (6). Fibulins have been shown to modulate cell morphology, growth, adhesion and motility, and are closely associated with the development of a wide variety of carcinomas (7). As tumor-suppressor genes, fibulin-2 (8,9) and fibulin-5 (10-12) were widely considered to be associated with the suppression of tumor growth, invasion and angiogenesis. Research findings on the role of fibulin-1 and fibulin-3 in different tumor tissues have been controversial. Few researchers have reported oncogenic activities (13-19), whereas others have reported tumor-suppressive activities (20-27). This discrepancy may be attributable to the influence of the tumor microenvironment on tumor-associated genes in promoting angiogenesis and metastasis (28).

Fibulin-4 is essential for connective tissue development and elastic fiber formation and may also play an important role in vascular patterning and collagen biosynthesis (29). Fibulin-4 plays a role in many clinical conditions such as cutis laxa (30), aortic aneurysms (31), osteoarthritis (32) and cancer (22,33). In their study on colon tumors, Gallagher *et al* found that the fibulin-4 gene was localized on chromosome 11q13 (33); translocations, amplifications and other rearrangements in this

region are associated with a variety of human cancers (34,35). Reverse transcriptase (RT)-polymerase chain reaction (PCR) of RNA from paired human colon tumors and adjacent normal tissue showed that tumors had a 2-7 fold increase in the level of fibulin-4 mRNA expression (33). However, in prostate cancer (22), fibulin-4 was found to be significantly downregulated and weakly expressed in carcinoma cell lines compared to normal prostate epithelial cells. Against this background of controversies in the research addressing the role of fibulin-4, more studies are needed to elucidate the relationship between fibulin-4 and cancer. To our knowledge, the role of fibulin-4 in cervical cancer remains unexplored.

The purpose of this study was to assess whether fibulin-4 expression is associated with the progression of cervical cancer and to investigate the relationship between fibulin-4 and angiogenesis.

Materials and methods

Cell lines. Highly invasive subclones (HeLa-1 and SiHa-1) and low-invasive subclones (HeLa-25 and SiHa-23) were derived from the HeLa and SiHa human cervical cancer cell lines, using the limited dilution method. Next, the cell electrophoretic mobility (EPM) of each clone was measured to study the charge-related properties using microcapillary electrophoresis chips. Finally, the MTT assay, soft agar colony formation assay, Matrigel invasion assay and cell migration assay were performed and tumor xenografts were generated in nude mice to confirm that highly invasive subclones and low-invasive subclones had high and low metastatic potential, respectively (3). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (Gibco-BRL, Rockville, MD, USA).

Tissue specimens. A total of 270 human cervical tissue specimens obtained with written informed consent from patients were used for this study. Two hundred and thirty cervical cancer patients were enrolled from the Department of Gynecology and Obstetrics, Shandong Provincial Hospital between 2006 and 2010. There were 60 cervical intraepithelial neoplasia (CIN) cases [age range, 28-55 years; mean (SD), 40 (8)], 140 squamous cell carcinoma cases [age range, 30-65 years; mean (SD), 45 (10)] and 30 adenocarcinoma cases [age range, 35-60; mean (SD), 43 (6)]. All cervical cancer patients were clinically staged according to the revised International Federation of Gynecology and Obstetrics (FIGO) staging system (FIGO stage I, 76 cases; FIGO stage II, 81 cases; and FIGO stage III and IV, 13 cases). None of the cervical cancer patients received preoperative radiation or chemotherapy. All patients were treated consecutively and were followed up regularly; 9 patients were lost to follow-up and 25 patients died during the study period. Follow-up duration was between 2 and 7 years by the end of 2012. Forty normal cervical tissue specimens [age range, 30-60 years; mean (SD), 47 (9)] were obtained from the Department of Gynecology and Obstetrics, Shandong Provincial Hospital. The study was approved by the Institutional Medical Ethics Committee of Shandong University.

Blood samples. Blood samples were obtained with written informed consent from the same 230 cervical cancer patients (60 CIN cases, 140 squamous cell carcinoma cases and 30 adenocarcinoma cases) at the Department of Gynecology and Obstetrics, Shandong Provincial Hospital between 2006 and 2010. None of the cervical cancer patients received preoperative radiation or chemotherapy. Forty control blood samples were obtained with written informed consent from age-matched examinees undergoing health examinations at Shandong Provincial Hospital. Control subjects had no history of disease and no abnormalities on laboratory examinations. The study was approved by the Institutional Medical Ethics Committee of Shandong University.

Enzyme-linked immunosorbent assay. Levels of fibulin-4 in serum samples were measured using sandwich enzyme-linked immunosorbent assay (ELISA) with human fibulin-4 ELISA assay kits (Immuno-Biological Laboratories, Fujioka, Gunma, Japan). Serum was diluted with enzyme immunoassay (EIA) buffer (1% bovine serum albumin, 0.05% Tween-20 in phosphate buffer) and incubated for 2 h at 37˚C. After 4 washes with EIA buffer, horseradish peroxidase-conjugated antibodies were added and incubated for 30 min at 4˚C. After 4 washes, 100 μl of tetramethylbenzidine solution was added and incubated for 30 min at room temperature. The reaction was stopped with 100 μl of 1 N sulfuric acid and measured using the ELISA reader at 450 nm.

Immunohistochemistry. According to standard streptavidin-biotin-peroxidase complex procedures, immunohistochemistry (IHC) was performed on formalin-fixed, paraffin-embedded sections (5-μm thick) and cell slides were fixed in 4% paraformaldehyde. Briefly, after dewaxing, rehydration, and antigen retrieval, the sections were incubated with anti-human fibulin-4 antibodies (ab125073; Abcam and MAB2644; Millipore) with working dilutions of 1:200 for ab125073 and 1:500 for MAB2644 at 4˚C overnight, and stained with the enzyme substrate 3',3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA). Human breast cancer paraffin-embedded sections (fibulin-4-positive) were used as positive controls. A negative control was obtained by replacing the primary antibody with normal rabbit or mouse immunoglobulin (IgG). Positive expression of fibulin-4 protein was defined as the presence of brown granules in the cytoplasm.

Immunohistochemistry analysis. A semi-quantitative scoring system derived from the method by Soumaoro *et a*l (36) for both the intensity of staining and the percentage of positive cells was used to evaluate fibulin-4 expression. The intensity of fibulin-4-positive staining was scored from 0 to 3 (negative, 0; weak, 1; moderate, 2; or strong, 3), and the percentage of positively stained cells was scored as 0 (0%), 1 (1-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%). The sum of the intensity and percentage scores was used as the final staining score (0-7). The sum-indices (-), (+), (++), and (+++) indicated final staining scores of 0, 1-3, 4-5 and 6-7, respectively. For statistical analysis, sum-indices (-) and (+) were defined as low fibulin-4 expression, while sum-indices (++) and (+++) were defined as high fibulin-4 expression. Each section was independently scored by two pathologists. In cases of an inconsistency, a third pathologist was consulted to arrive at a consensus. To assess reproducibility, we invited three other pathologists to

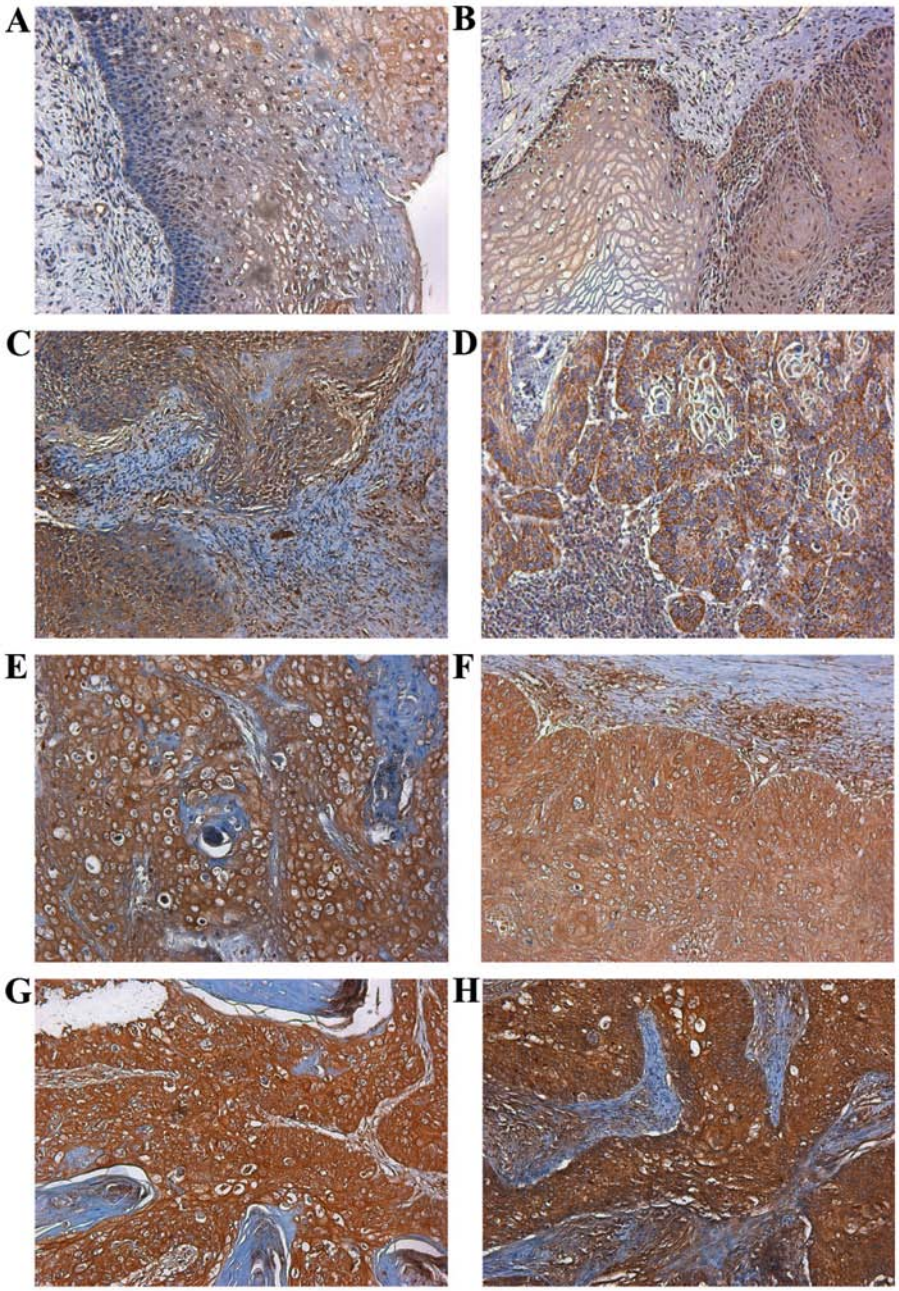

Figure 1. Expression of fibulin-4 in human cervical tissues with the ab125073 antibody. (A) Normal human cervical tissue, (B) cervical intraepithelial neoplasia (CIN), (C and D) stage I cervical carcinoma, (E and F) stage II cervical carcinoma, (G and H) stage III and IV cervical carcinoma (magnification, x200).

score all sections independently. The interobserver reliability and intraobserver reproducibility of IHC experiments were evaluated using kappa (κ) statistical evaluation.

Microvessel assessment. Microvessel density (MVD) was assessed according to CD34 immunohistochemical staining of tumor vessels. Any immune-positive single endothelial cell or endothelial cell clusters and microvessels in the tumor were considered to be individual vessels and were counted, as described by Weidner *et al* (37). Peritumoral vascularity, vascularity in areas of necrosis, and vessels with a thick muscle wall or having a diameter larger than 8 erythrocytes, were not counted. The sections were scanned at low power (x100) to select the most vascularized (hot-spots) areas. The microvessels in the hot-spots were then counted, and an average count in three hot spots was calculated as the MVD. All counts were performed independently by three observers who were blinded to the corresponding clinicopathological data.

Quantitative real-time-polymerase chain reaction. Total RNA was extracted using TRIzol reagent (Invitrogen) and reverse transcribed. Quantitative real-time RT-PCR analysis was performed using ABI Prism 7500 Real-Time PCR System (Applied Biosystems). Each well (20-μl reaction volume) contained 10 μl Power SYBR-Green PCR Master Mix (Applied Biosystems), 1 μl of each primer (5 μmol/l) and 1 μl template. The following primers were used: fibulin-4, 5'-GCTGCTACT GTTGCTCTTGGG-3' and 5'-GGGATGGTCAGACACTCGT TG-3'; β-actin 5'-CCACGAAACTACCTTCAACTCCA-3' and 5'-GTGATCTCCTTCTGCATCCTGTC-3'.

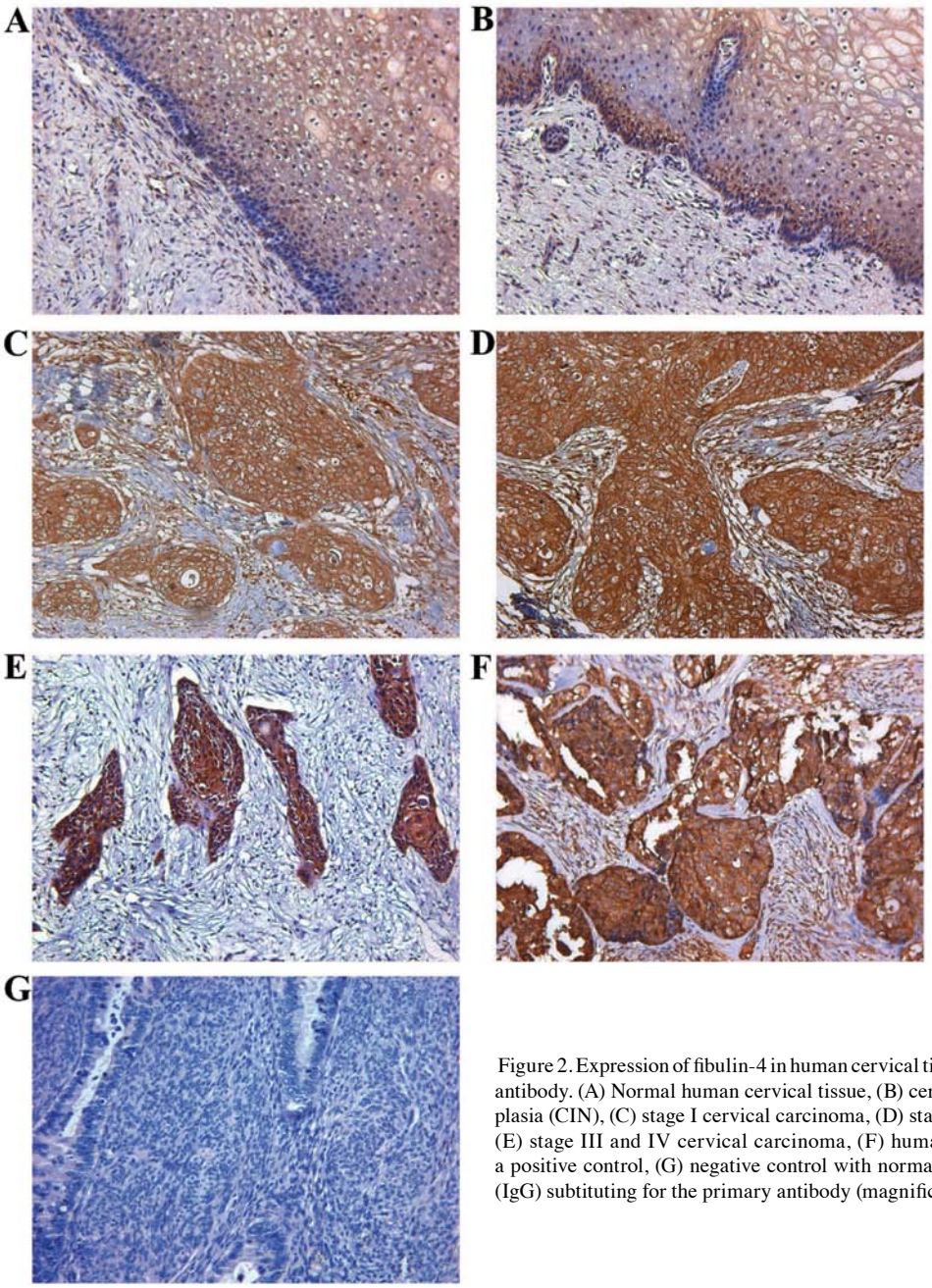

Figure 2. Expression of fibulin-4 in human cervical tissues with the MAB2644 antibody. (A) Normal human cervical tissue, (B) cervical intraepithelial neoplasia (CIN), (C) stage I cervical carcinoma, (D) stage II cervical carcinoma, (E) stage III and IV cervical carcinoma, (F) human breast cancer used as a positive control, (G) negative control with normal rabbit immunoglobulin (IgG) subtituting for the primary antibody (magnification, x200).

Statistical analysis. IHC data were analyzed using the Chi-square test. Measurement data were expressed as means ± SE. The interobserver reliability and intraobserver reproducibility of IHC experiments were evaluated using the κ statistical evaluation. The strength of agreement was interpreted as follows: excellent (κ ≥0.80), good (0.60-0.79), moderate (0.40-0.59), poor (0.20-0.39) and very poor (<0.20) (38). For comparison of means between two groups, a two-tailed t-test was used and for comparison of means among three groups, one-way ANOVA was used. Survival curves were calculated using the Kaplan-Meier method and analyzed using the log-rank test. Correlations of fibulin-4 expression with VEGF expression and MVD were analyzed using the Pearson correlation test. Multivariate Cox proportional hazards models were used to define the potential prognostic significance of individual parameters. Statistical analysis was performed using SPSS software version 13.0. Two-sided P-values of <0.05 were considered to indicate statistically significant differences.

Results

Fibulin-4 expression in the human cervical tissues. Fibulin-4 protein expression was extremely low in the normal human cervical tissue and CIN (Figs. 1A and B; 2A and B). However, in most cervical carcinomas, fibulin-4 immunoreactivity was high, and high fibulin-4 protein expression was detected in the cytoplasm of cervical cancer cells (Figs. 1C-H; 2C-E). Moreover, high fibulin-4 protein expression was associated with low differentiation, advanced stage and positive lymph node status of the cervical carcinoma cases (Tables I and II). The interobserver reliability coefficients were 0.86 and 0.81

Table I. Protein expression of fibulin-4 in the human cervical tissues with the ab125073 antibody.

		Fibulin-4 low (-/+)		Fibulin-4 high (++/+++)			
	N	n	(%)	n	(%)	χ^2	P-value
Normal	40	37	(92.5)	3	(7.5)	66.05	<0.01
CIN	60	44	(73.3)	16	(26.7)		
Carcinoma	170	53	(31.2)	117	(68.8)		
Pathological type						0.02	>0.05
Squamous cell carcinoma	140	44	(31.4)	96	(68.6)		
Adenocarcinoma	30	9	(30.0)	21	(70.0)		
Cell differentiation						25.57	<0.01
High and medium	89	43	(48.3)	46	(51.7)		
Low	81	10	(12.3)	71	(87.7)		
Tumor stage						26.07	<0.01
I	60	32	(53.3)	28	(46.7)		
II	59	19	(32.2)	40	(67.8)		
III and IV	51	4	(7.8)	47	(92.2)		
Nodal status						18.26	<0.01
Positive	66	8	(12.1)	58	(87.9)		
Negative	104	45	(43.3)	59	(56.7)		

CIN, cervical intraepithelial neoplasia.

Table II. Protein expression of fibulin-4 in the human cervical tissues with the MAB2644 antibody.

		Fibulin-4 low (-/+)		Fibulin-4 high (++/+++)			
	N	n	(%)	n	(%)	χ^2	P-value
Normal	40	36	(90)	4	(10)	63.468	<0.01
CIN	60	42	(70)	18	(30)		
Carcinoma	170	50	(29.4)	120	(70.6)		
Pathological type						0.270	>0.05
Squamous cell carcinoma	140	40	(28.6)	100	(71.4)		
Adenocarcinoma	30	10	(33.3)	20	(66.7)		
Cell differentiation						21.705	<0.01
High and medium	89	40	(44.9)	49	(55.1)		
Low	81	10	(12.3)	71	(87.7)		
Tumor stage						32.759	<0.01
I	76	39	(51.3)	37	(48.7)		
II	81	11	(13.6)	70	(86.4)		
III and IV	13	0	(0)	13	(100)		
Nodal status						24.525	<0.01
Positive	66	6	(9.1)	60	(90.9)		
Negative	104	47	(45.2)	57	(54.8)		

CIN, cervical intraepithelial neoplasia.

for the first and second assessments, with an intraobserver reproducibility coefficient of 0.85. The interobserver reliability and intraobserver reproducibility of the IHC experiments were excellent. Similarly results were also found for the real-time

Table III. mRNA expression of fibulin-4 in the human cervical tissues.

	N	Fibulin-4 mRNA	P-value
Control	40	0.0096±0.0064	
CIN	60	0.0091±0.0048	>0.05[a]
Carcinoma	170	0.0769±0.0089	<0.05[b]
Pathological type			>0.05
Squamous cell carcinoma	140	0.0648±0.0115	
Adenocarcinoma	30	0.0796±0.0127	
Cell differentiation			<0.05
High and medium	89	0.0284±0.0078	
Low	81	0.0932±0.0105	
Tumor stage			<0.05
I	76	0.0267±0.0073	
II	81	0.0541±0.0081	
III and IV	13	0.0946±0.0126	
Nodal status			<0.05
Positive	66	0.0979±0.0117	
Negative	104	0.0243±0.0059	

[a]CIN compared with healthy control, P>0.05; [b]cervical carcinoma compared with healthy control and CIN, P<0.05. CIN, cervical intraepithelial neoplasia.

Table IV. Predictive factors of survival by multivariate analysis (Cox proportional hazards model).

Prognostic factors	HR (95% CI)	P-value
Fibulin-4	1.019 (1.007-1.031)	0.002
Pathological type	0.978 (0.429-2.230)	0.957
Cell differentiation	1.012 (0.999-1.026)	0.065
Tumor stage	3.175 (1.361-7.403)	0.007
Lymph node metastasis	2.129 (1.319-3.435)	0.002
Tumor size	1.095 (0.986-1.216)	0.089
Age (years)	0.981 (0.957-1.005)	0.125

HR, hazard ratio; CI, confidence interval.

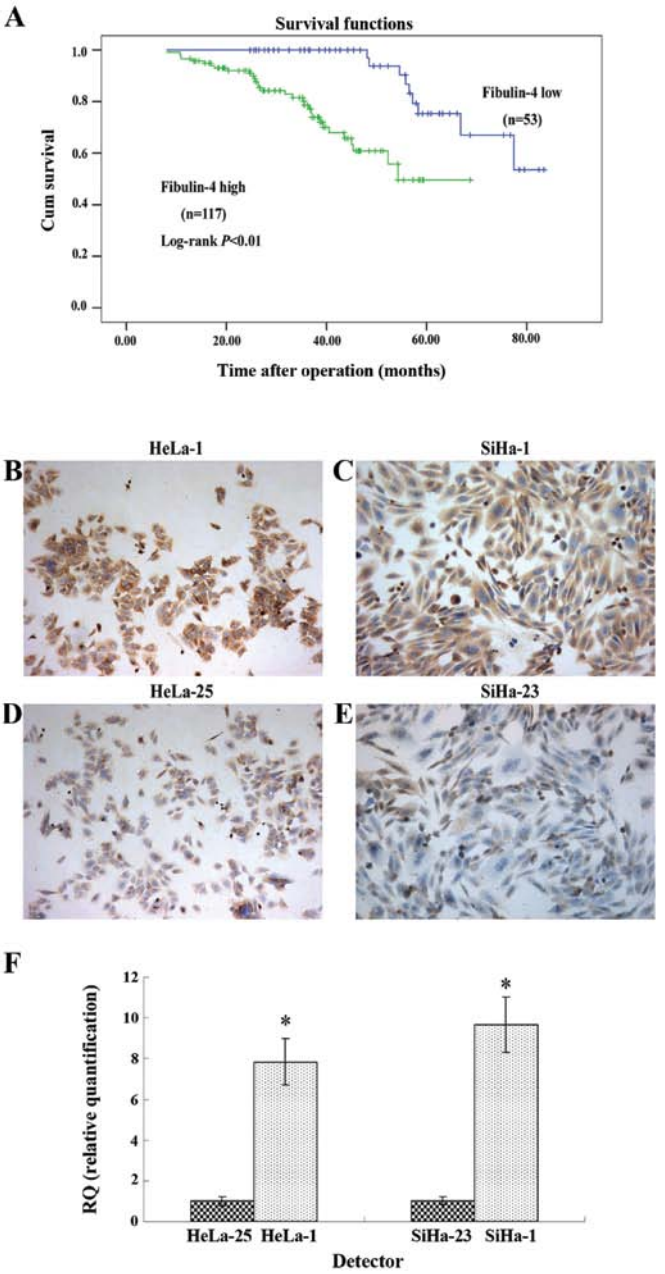

Figure 3. Kaplan-Meier analysis and fibulin-4 expression in the highly invasive subclones and in the low-invasive subclones. (A) Kaplan-Meier analysis of the overall survival of the patients whose tumors had high or low fibulin-4 expression. (B-E) Fibulin-4 protein expression in the highly invasive subclones (B) HeLa-1 and (C) SiHa-1 and low-invasive subclones (D) HeLa-25 and (E) SiHa-23 as measured by IHC staining (magnification, x200). (F) Fibulin-4 mRNA expression in the highly invasive subclones HeLa-1 and SiHa-1 and low-invasive subclones HeLa-25 and SiHa-23 as measured by qRT-PCR. *P<0.05 vs. control.

RT-PCR experiment; fibulin-4 mRNA expression was also extremely low in the normal cervical tissues and CIN, and significantly high fibulin-4 expression was noted in the cervical carcinoma cases. Moreover, high fibulin-4 mRNA expression was also associated with low differentiation, advanced stage and positive lymph node status of the cervical carcinomas (Table III). To evaluate the prognostic value of fibulin-4 in cervical cancer, we performed survival analysis using Kaplan-Meier analysis. The results showed that patients with high fibulin-4 expression had a much worse prognosis than those with low fibulin-4 expression (log-rank, P<0.01) (Fig. 3A). In the multivariate analysis, considering all histological and molecular features together, the significant prognostic factors were lymph node metastasis (P=0.002; hazard ratio 2.129), fibulin-4 expression (P=0.002; hazard ratio 1.019) and tumor stage (P=0.007; hazard ratio 3.175) (Table IV).

Differential expression of fibulin-4 in the highly invasive subclones and the low-invasive subclones. The highly invasive subclones (HeLa-1 and SiHa-1) and the low-invasive subclones (HeLa-25 and SiHa-23) were derived from the HeLa

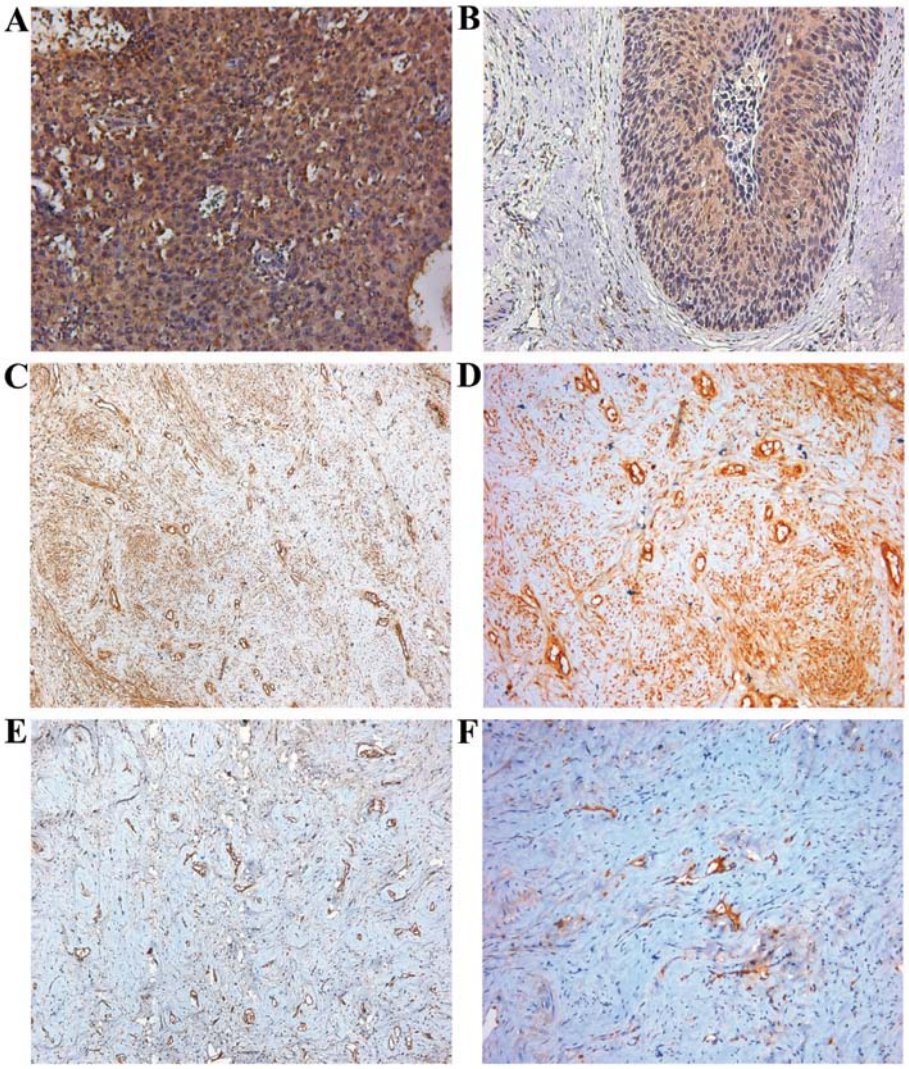

Figure 4. Immunohistochemical staining of VEGF and CD34 for determination of microvessel density (MVD). Immunohistochemical staining of VEGF in stages IV cervical carcinoma (A), and stage I cervical carcinoma (B) (magnification, x200). Immunohistochemical staining of CD34 for MVD in stage IV cervical carcinoma (C, x100 magnification) (D, x200 magnification), and in stage I cervical carcinoma (E, x100 magnification) (F, x200 magnification).

and SiHa human cervical cancer cell lines, using the limited dilution method. Since the cell lines have similar genetic backgrounds, they are suitable for comparative analysis. As shown in Fig. 3B-F, fibulin-4 protein and mRNA expression levels were extremely high in the highly invasive subclones (HeLa-1 and SiHa-1), compared to the low-invasive subclones (HeLa-25 and SiHa-23).

Serum levels of fibulin-4 in human cervical cancer patients and healthy control. As shown in Table V, the serum fibulin-4 level in cervical carcinoma patients was much higher than that in the healthy controls and CIN patients ($P<0.05$). No significant difference was found between healthy controls and CIN ($P>0.05$). Moreover, high serum levels of fibulin-4 were associated with low differentiation, advanced stage and positive lymph node status of the cervical carcinoma cases ($P<0.05$). There were no significant differences among the different pathological types of cervical carcinoma ($P>0.05$).

Relationships of fibulin-4 with VEGF expression and MVD. Fig. 4 shows the representative immunohistochemical staining images for VEGF and CD34. The immunohistochemical expression of VEGF and fibulin-4 was evaluated using software Image-Pro Plus 6.0 to detect photodensity. In brief, five positive fields in a section were selected at random and then read using Image-Pro Plus 6.0, and the average densities were then calculated. Pearson correlation tests of MVD (Fig. 5A, $P<0.01$) and VEGF expression (Fig. 5B, $P<0.01$) vs. fibulin-4 revealed strong positive correlations.

Discussion

In the present study, we demonstrated for the first time that the expression of fibulin-4 is associated with poor prognostic clinicopathologic features, neovascularization and poor outcome in human cervical carcinoma patients.

Our immunohistochemical studies showed an upregulation of fibulin-4 expression in cervical carcinoma tissues, compared with normal cervical tissues and CIN. Fibulin-4 is rich in elastic fiber tissue, and is mainly involved in the synthesis and arrangement of elastic fibers (39). In the present study, the expression of fibulin-4 in the extracellular matrix was found

Table V. Serum levels of fibulin-4 in the patients with cervical tumors.

	N	Fibulin-4 (ng/ml)	P-value
Control	40	108.43±11.86	
CIN	60	116.57±13.24	>0.05[a]
Carcinoma	170	356.49±22.15	<0.05[b]
Pathological type			>0.05
Squamous cell carcinoma	140	267.26±16.54	
Adenocarcinoma	30	271.32±18.61	
Cell differentiation			<0.05
High and medium	89	106.93±10.22	
Low	81	347.56±23.47	
Tumor stage			<0.05
I	76	109.84±13.51	
II	81	213.47±15.69	
III and IV	13	368.51±24.85	
Nodal status			<0.05
Positive	66	357.34±20.06	
Negative	104	113.92±13.43	

[a]CIN compared with healthy control, P>0.05; [b]cervical carcinoma compared with healthy control and CIN, P<0.05. CIN, cervical intraepithelial neoplasia.

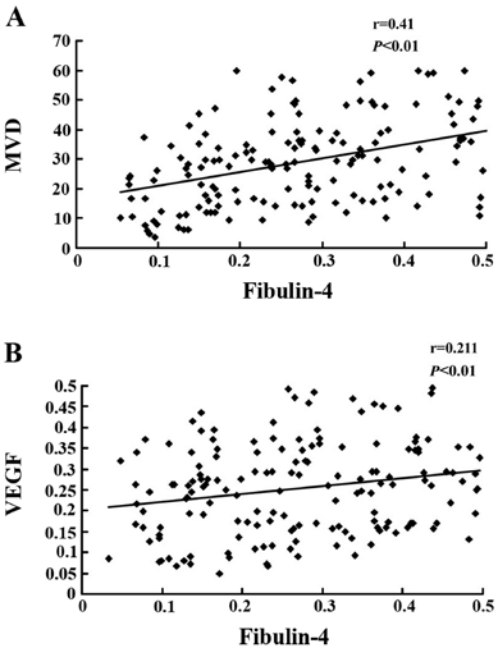

Figure 5. Pearson correlation analysis of fibulin-4 expression and MVD and VEGF. The expression of fibulin-4 was positively correlated with (A) MVD and (B) VEGF.

to be much less than that in the cancer cell cytoplasm, which probably was due to the presence of fewer elastic fibers in cervical tissue. In the basement membranes, however, we found stronger expression of fibulin-4 in CIN when compared with the normal tissue. Real-time RT-PCR experiments confirmed that mRNA expression of fibulin-4 was also upregulated in the cervical carcinoma tissues. Moreover, high fibulin-4 expression was associated with low differentiation, advanced stage and positive lymph node status in the cervical carcinomas. Similar results have been reported in earlier studies on colon cancer; dysregulated expression of the fibulin-4 gene was shown to be associated with human colon tumorigenesis (33). However, contrasting results have also been reported for prostate cancer. By microarray analysis, the fibulin-4 genes were found to be significantly downregulated in prostate cancer and this result was corroborated by qRT-PCR (22). In this study, fibulin-4 was overexpressed in cervical cancer and was shown to play an important role in tumor development. As is the case for other fibulins, there are controversies in research on fibulin-4; these discrepancies may be attributable to the fact that the tumor microenvironment influences the functions of tumor-associated genes.

Angiogenesis is the process of formation of new microvessels from preexisting vasculature. Once the tumor volume exceeds a few millimeters in diameter, hypoxia and nutrient deprivation trigger tumor cells to exploit their microenvironment by releasing cytokines and growth factors, which then activate normal, quiescent cells around them and initiate a cascade of events resulting in tumor progression. For example, tumor cell-derived VEGF stimulates the sprouting and proliferation of endothelial cells. VEGF is considered the most potent candidate for angiogenesis induction during tumor growth (40). Since angiogenesis is essential for tumor growth and metastasis, controlling tumor-associated angiogenesis is a promising strategy for inhibiting cancer progression. In our study, we sought to determine whether fibulin-4 is associated with angiogenesis. To this end, the Pearson correlation coefficient was calculated to assess the correlation of fibulin-4 with MVD and VEGF expression. We found that fibulin-4 expression was positively correlated with MVD and VEGF expression, which indicated that fibulin-4 may promote angiogenesis. No previous studies concerning fibulin-4 have reported an association with tumor angiogenesis, although its highly homologous proteins, fibulin-3 and fibulin-5 were found to be associated with tumor angiogenesis. Exogenous and endogenous fibulin-5 were shown to be anti-angiogenic (41). Fibulin-3 was initially found to exert an anti-angiogenic effect (42), but in recent years, several studies have reported that fibulin-3 can promote angiogenesis, particularly in pancreatic adenocarcinoma and cervical cancer. Fibulin-3 gene transfection elevates VEGF expression and MVD (16,17). Since fibulin-4 is highly homologous to fibulin-3 and fibulin-5, we speculate that fibulin-4 may play a significant role in tumor angiogenesis. Pearson correlation tests of MVD and VEGF expression versus the corresponding expression of fibulin-4 revealed strong direct correlations. Hence, we conclude that fibulin-4 may promote cervical tumor angiogenesis. However, further studies are needed to confirm our speculation, including cell transfection experiments, chorioallantoic membrane assays and tumor xenograft models in nude mice.

High serum levels of fibulin-4 were found in cervical carcinoma patients when compared with healthy controls and CIN patients, and high fibulin-4 levels were associated with

low differentiation, advanced stage, and positive lymph node status in cervical carcinomas. This discovery may aid in determining the diagnosis and prognosis of cervical carcinoma. In recent years, fibulins have been recognized as biomarkers for many diseases, such as osteoarthritis, pleural mesothelioma and breast carcinoma. Fibulin-3 and fibulin-4 may play pathogenic roles in osteoarthritis (32,43). The plasma fibulin-3 and fibulin-1 levels were found to be elevated in patients with mesothelioma and breast carcinoma, respectively (44,45). Novel specific biomarkers can help detect diseases at an earlier stage and tailor treatment strategies for individualized management. Fibulin-4 may be exploited as a tool for the early detection of cervical carcinoma.

In conclusion, fibulin-4 is a newly identified gene that is overexpressed in cervical carcinoma, promotes angiogenesis, and is associated with poor prognosis. Serum levels of fibulin-4 may be helpful in early diagnosis and determining the prognosis in cases of cervical cancer. Fibulin-4 may also serve as a novel therapeutic target in patients with cervical carcinoma.

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