Clin. Lab. 2018;64:XXX-XXX

ORIGINAL ARTICLE

Fibulin-5 is Down-Regulated in Colorectal Cancer and Correlated with Clinicopathologic Characteristics

Zhilei Chen, Zhenjun Wang, Jianjun Chen, Zhulin Li

Department of General Surgery, Beijing Chao-Yang Hospital, Capital Medical University, Chaoyang District, Beijing, China

SUMMARY

Background: **Fibulin-5 has recently been considered as a potential tumor suppressor in human cancers. Several studies have shown that it is down-regulated in a variety of tumor types and inhibits tumor growth and metastasis. In this study, the expression of fibulin-5 in colorectal cancer (CRC) and its clinical significance were assessed.**
Methods: **Fibulin-5 expression was detected in 31 samples of surgically resected CRC and paired noncancerous tissues using western blot, qRT-PCR, and immunoblotting.**
Results: **In this study, the expression levels of fibulin-5 protein and mRNA were down-regulated in CRC tissues as compared with those in paired noncancerous tissues. Low expression of fibulin-5 was significantly correlated with poor prognostic features including lymph node metastasis and advanced tumor-node-metastasis (TNM) tumor stage.**
Conclusions: **Fibulin-5 is down-regulated in CRC and the reduced expression of fibulin-5 was correlated with malignant clinicopathological characteristics.**
(Clin. Lab. 2018;64:xx-xx. DOI: 10.7754/Clin.Lab.2017.170519)

Correspondence:
Prof. Zhenjun Wang
Department of General Surgery
Beijing Chao-Yang Hospital
Capital Medical University
No. 8 Gongti Nan Lu
Chaoyang District, Beijing 100020
China
Phone: +86 10-85231604
Fax: +86 10-88340286
Email: drzhenjun@163.com

KEY WORDS

fibulin-5, colorectal cancer, clinical significance, tumor suppressor

LIST OF ABBREVIATIONS

CRC - colorectal cancer
TNM - tumor-node-metastasis
ECM - extracellular matrix
RGD - Arg-Gly-Asp
qRT-PCR - real-time quantitative reverse transcription polymerase chain reaction
MMP-7 - matrix metalloproteinase-7
TGF-β - transforming growth factor-beta
EMT - epithelial-mesenchymal transition
VEGF - vascular endothelial growth factor
MYC - myelocytomatosis oncogene

Manuscript accepted September 3, 2017

INTRODUCTION

Colorectal cancer (CRC), one of the most common malignant tumors in the digestive system, results in approximately 690,000 deaths annually around the word [1]. Tumor resection followed by adjuvant and neoadjuvant is the current standard therapy for CRC. Due to its expanded therapy and the need for lifelong surveillance, CRC is also one of the most expensive cancers to treat [2]. However, the prognosis of patients with CRC remains poorly understood. Therefore, the research on prognostic markers or potentially valuable tumor suppressor genes should be carried out urgently.

The fibulin family, which comprises fibulin-1 to -7, is an old protein family discovered in both worms and humans. Fibulin-5, a 66 kDa glycoprotein, is produced by various cell types and is essential for the formation of elastic fibers [3]. Similar to the functions of other fibulin family members, fibulin-5 plays important roles in cell-to-cell and cell-to-matrix communication and helps to organize and stabilize extracellular matrix (ECM) structures during organogenesis and vasculogenesis [4]. In addition, fibulin-5 contains an integrin-binding Arg-Gly-Asp (RGD) motif which binds to integrins and mediates endothelial cell adhesion [5]. Previous studies have shown that it is down-regulated in a variety of tumor types, including breast, ovarian [6], hepatocellular [7], prostate [8], bladder [9], and lung [10] cancers. However, this contrasts with findings in human fibrosarcoma [11] and gastric [12] cancers, suggesting that the actions of this ECM protein are tissue-dependent. The role of fibulin-5 in human CRC has not been reported previously. We hypothesized that fibulin-5 expression may be reduced in human CRC, as in most of the other epithelial malignancies. The present study aimed to determine the expression and localization of fibulin-5 in human CRC across different tumor stages and pathological types. Furthermore, we may disclose that fibulin-5 acts as a valuable tumor marker and inhibits tumor progression in CRC.

MATERIALS AND METHODS

Patients and tissue samples

A total of 31 CRC patients including 23 males and 8 females, who received radical operation in the Department of General Surgery at Beijing Chao-yang Hospital from June 2016 to December 2016 (Table 1), were included. CRC samples and paired normal tumor-adjacent samples (> 5 cm distance from the margin of the tumor) were collected during the surgery. Fresh tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C. This study was approved by the ethics committee of Beijing Chao-yang Hospital, and informed consent was obtained from each patient before surgery.

Western blot

Fibulin-5 (ab66339, abcam, Cambridge, UK) (1:1000) and GAPDH (5174, cst, MA, USA) (1:1000) antibodies were used for immunoblotting assay. Horseradish peroxidase (HRP)-conjugated sheep anti-rabbit secondary antibody (305-035-003, Jackson, CA, USA) were used at a dilution 1:10000 and detected by Millipore ECL (WBKLS0500, Millipore, MA, USA). The intensities of the bands of interest were expressed relative to the GAPDH intensities from the same sample and quantified by Gel Image system ver. 4.00 software (Tanon, China).

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The primer sequences were as follows:
fibulin-5 forward,
5'-TCGCTATGGTTACTGCCAGCA-3';
fibulin-5 reverse,
5'-TTGGCAAGACCTTCCATCGTC-3';
GAPDH forward,
5'-AGAAGGCTGGGGCTCATTTG-3';
GAPDH reverse,
5'-AGGGGCCATCCACAGTCTTC-3'.
Total RNA was isolated from tissues using TRIZOL® reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthetized using the TIANScript RT Kit (TIANGEN, Beijing, CN). The PCR amplification for the quantification of the fibulin-5 mRNA and the GAPDH mRNA was performed using an Applied Biosystems 7500HT Fast Block Real Time PCR system (Thermo Fisher Scientific, Inc). The GAPDH was used as an internal control. All PCR reactions were run in triplicate, and gene expression relative to GAPDH was calculated using the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemical staining

Formalin-fixed paraffin-embedded tissue sections were subjected to antigen retrieval by treatment with 0.01 M Tris-HCl (pH 9.0), followed by incubation with anti-fibulin-5 (ab202977, abcam, Cambridge, UK). Positively stained areas were measured by average optical density using Image Pro Plus (Media Cybernetics, USA).

Statistical analysis

Results are expressed as mean ± SEM. Significance was established, with the SPSS statistical package for Windows Version 13 (SPSS, Chicago, IL, USA), using a Pearson's chi-squared test, a two-tailed Student's *t*-test, a Mann-Whitney test or a Pearson's correlation coefficient when appropriate. Differences were considered significant when $p < 0.05$.

Table 1. Summary of clinical data.

Patient no.	Years	Gender	Histologic type	Tumor location	TNM Tumor stage	Group
1	68	Male	Adenocarcinoma	Sigmoid colon	pT4aN0Mx	IIB
2	61	Male	Mucinous adenocarcinoma	Rectum	pT3N0Mx	IIA
3	67	Male	Adenocarcinoma	Sigmoid colon	pT2N0Mx	I
4	69	Male	Mucinous adenocarcinoma	Transverse colon	pT3N1bMx	IIIA
5	75	Female	Adenocarcinoma	Sigmoid colon	pT4N0Mx	IIB
6	75	Male	Adenocarcinoma	Rectum	pT3N1Mx	IIIA
7	76	Female	Mucinous adenocarcinoma	Ascending colon	pT3N0Mx	IIA
8	84	Female	Mucinous adenocarcinoma	Transverse colon	pT3N1Mx	IIIA
9	68	Male	Adenocarcinoma	Transverse colon	pT3N1Mx	IIIA
10	66	Male	Adenocarcinoma	Sigmoid colon	pT3N1Mx	IIIA
11	71	Male	Adenocarcinoma	Rectum	pT3N2Mx	IIIB
12	50	Male	Adenocarcinoma	Sigmoid colon	pT3N1Mx	IIIA
13	63	Female	Adenocarcinoma	Rectum	pT3N0Mx	IIA
14	59	Male	Adenocarcinoma	Rectum	pT3N1bMx	IIIA
15	73	Male	Adenocarcinoma	Descending colon	pT3N0Mx	IIA
16	61	Female	Adenocarcinoma	Rectum	pT3N0Mx	IIA
17	73	Male	Mucinous adenocarcinoma	Ascending colon	pT4N2bMx	IIIC
18	80	Female	Mucinous adenocarcinoma	Sigmoid colon	pT4N2bMx	IIIC
19	85	Male	Adenocarcinoma	Rectum	pT2N0Mx	I
20	68	Male	Adenocarcinoma	Ascending colon	pT3N0Mx	IIA
21	66	Male	Adenocarcinoma	Ascending colon	pT3N0Mx	IIA
22	68	Male	Mucinous adenocarcinoma	Sigmoid colon	pT3N1cMx	IIIA
23	59	Male	Mucinous adenocarcinoma	Transverse colon	pT3N0Mx	IIA
24	76	Male	Mucinous adenocarcinoma	Rectum	pT3N0Mx	IIA
25	63	Male	Adenocarcinoma	Rectum	pT3N0Mx	IIA
26	78	Female	Adenocarcinoma	Sigmoid colon	pT3N1bMx	IIIA
27	77	Female	Mucinous adenocarcinoma	Ascending colon	pT3N0Mx	IIA
28	72	Male	Adenocarcinoma	Rectum	pT1N0Mx	I
29	66	Male	Adenocarcinoma	Descending colon	pT3N0Mx	IIA
30	62	Male	Adenocarcinoma	Rectum	pT3N1Mx	IIIA
31	66	Male	Mucinous adenocarcinoma	Rectum	pT3N2bM1	IVC

TNM tumor stage was determined by postoperative pathological diagnosis.

RESULTS

The expression of fibulin-5 in CRC and paired noncancerous tissues

Initially, we tested fibulin-5 expression in 31 pairs of cancerous and paired noncancerous tissues using western blot. In these cases, we found that fibulin-5 expression in CRC tissues was prominently lower than that in paired noncancerous tissues (tumor: 0.297 ± 0.148 vs. nontumor: 0.406 ± 0.142, $p < 0.01$ by *t*-test, Figure 1). Furthermore, all of these CRC tissues and paired noncancerous tissues were subjected to qRT-PCR for fibulin-5 mRNA. The difference of fibulin-5 mRNA levels between cancer and paired noncancerous tissues were the same as protein levels (tumor mean rank: 26.71 vs. nontumor mean rank: 36.29, $p < 0.05$ by Mann-Whitney test, Figure 2). Finally, fibulin-5 was immunolocalized in these specimens. Fibulin-5 localized mostly to the stroma, with little staining in the glandular epithelium (Figure 3A). When staining intensity was measured by average optical density using Image Pro Plus, there was a significant reduction in CRC tissues compared to that in paired noncancerous tissues (tumor: 2.62 ± 2.17 vs. nontumor: 9.69 ± 6.12, $p < 0.01$ by *t*-test, Figure 3B).

Table 2. Correlation between the clinicopathologic characteristics and expression of fibulin-5 protein in CRC.

Characteristics		No. of patients	Fibulin-5	r value	p-value
Years	< 70	18	0.289 ± 0.158	0.064	0.731
	≥ 70	13	0.308 ± 0.137		
Gender	Male	23	0.292 ± 0.157	0.058	0.758
	Female	8	0.311 ± 0.123		
Tumor size (cm)	< 5	14	0.267 ± 0.116	0.181	0.330
	≥ 5	17	0.321 ± 0.169		
Histologic type	Adenocarcinoma	20	0.298 ± 0.152	-0.014	0.941
	Mucinous adenocarcinoma	11	0.294 ± 0.147		
Tumor location	Right of the colon	9	0.336 ± 0.157	-0.173	0.353
	Left of the colon or rectum	22	0.281 ± 0.144		
Lymph node metastasis	No	18	0.352 ± 0.146	-0.452	0.011 *
	Yes	13	0.220 ± 0.114		
TNM tumor stage	I + II	17	0.367 ± 0.137	-0.531	0.002 *
	III + IV	14	0.212 ± 0.114		
Serum CEA level (ng/mL)	< 5	23	0.292 ± 0.149	0.055	0.769
	≥ 5	8	0.310 ± 0.151		
BVI or LVI	No	17	0.337 ± 0.161	-0.309	0.091
	Yes	14	0.247 ± 0.116		

BVI - blood vessel invasion, LVI - lymphatic vessel invasion, * - statistically significant.

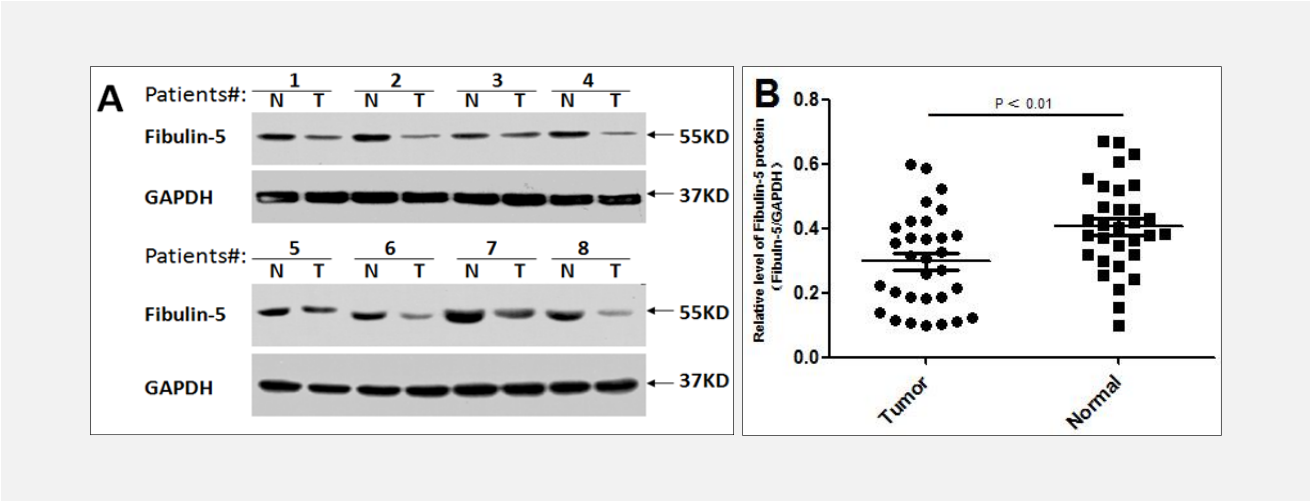

Figure 1. Expression of fibulin-5 protein in CRC.

A) Representative western blot analysis of fibulin-5 expression in the CRC (T) and paired noncancerous tissues (N) was shown. B) Quantification of the data revealed that fibulin-5 protein expression level in the cancer tissues was significantly lower than that in the paired noncancerous tissues. n = 31; p < 0.01 by *t*-test.

Thus, our data indicate that fibulin-5 is down-regulated in CRC tissues compared with controls.

Clinical significance of fibulin-5 expression in CRC tissues

To investigate the clinical significance of fibulin-5 in CRC, we analyzed the correlation between fibulin-5 ex-

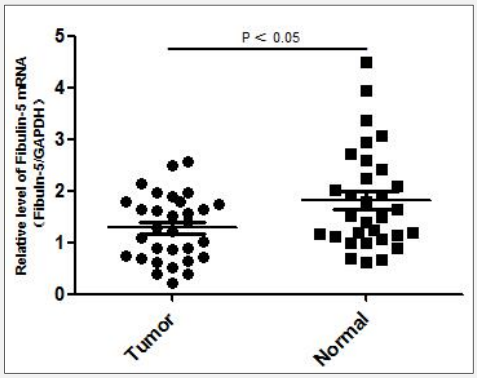

Figure 2. Expression of fibulin-5 mRNA in CRC.

Quantification of the data revealed that fibulin-5 mRNA level in the cancer tissues was significantly lower than that in the paired noncancerous tissues. n = 31; p < 0.05 by Mann-Whitney test.

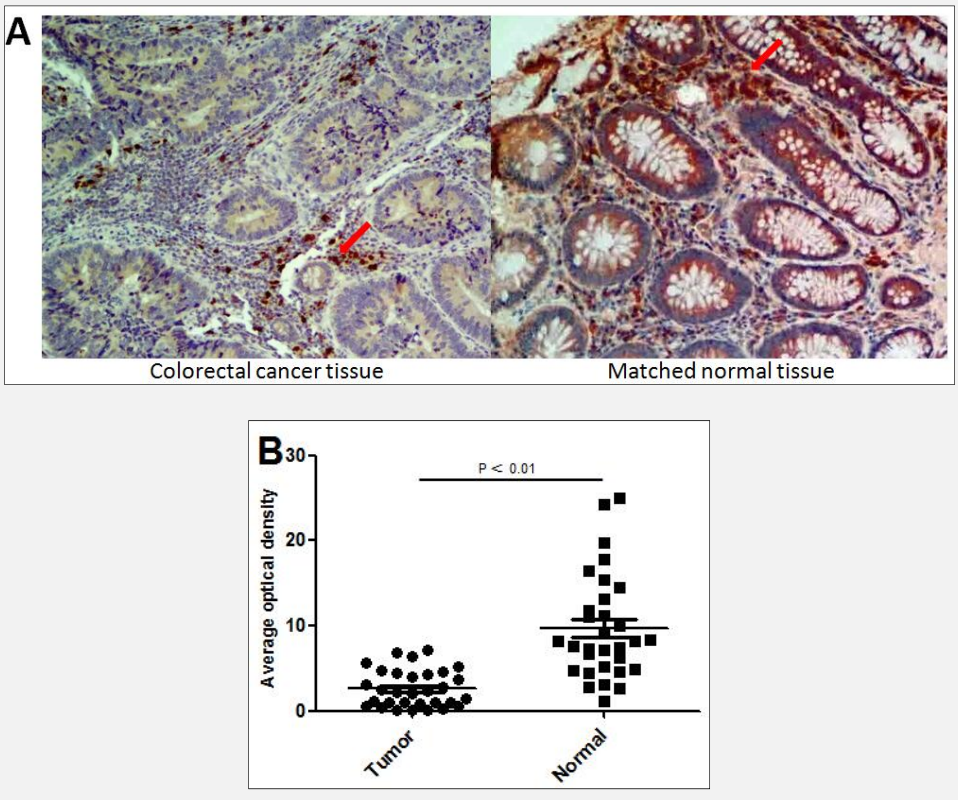

Figure 3. Expression of fibulin-5 immunohistochemistry staining in CRC.

A) Representative immunohistochemistry staining for fibulin-5 expression in the CRC (T) and paired noncancerous tissues (N) was shown. Fibulin-5 was immunolocalised mostly to the stroma, with little staining in the glandular epithelium. Representative photomicrographs are shown at x 100 magnification. Arrows denote positive stroma staining. B) Quantification of the data revealed that fibulin-5 average optical density in the cancer tissues was significantly lower than that in the paired noncancerous tissues. n = 31; p < 0.01 by *t*-test.

pression and clinicopathological parameters. As shown in Table 2, clinical association analysis using an Independent-Samples *t*-test and a Pearson's correlation coefficient indicated that the down-regulated expression of fibulin-5 in CRC was evidently correlated with lymph node metastasis ($r = -0.452$, $p = 0.011$) and advanced tumor-node-metastasis (TNM) tumor stage ($r = -0.531$, $p = 0.002$). Furthermore, low expression of fibulin-5 protein may be associated with blood vessel invasion or lymphatic vessel invasion, but not statistically significant ($r = -0.309$, $p = 0.091$). Our results indicate that the reduced expression of fibulin-5 was associated with malignant clinicopathological parameters in CRC.

DISCUSSION

Extracellular matrix (ECM), which is an important component of the tumor microenvironment, provides not only structural support but also physically contextual cell-to-cell and cell-to-matrix communications [13]. The proteins in the ECM are mostly glycoproteins, which include relatively large molecules such as laminins, fibronectins, and elastins. However, smaller ECM proteins like the fibulin family are more important in modulating cell behavior and functions [14].

The fibulins interact with many kinds of ECM components and are involved in fibrogenesis, tissue organogenesis, vasculogenesis, and tumorigenesis [15]. Fibulin-5, found in 1999, is produced by various cell types and is essential for the formation of elastic fibers. Fibulin-5 is initially found to be expressed in the neural crest and embryonic vasculature, while it is impaired in most adult vascular beds [16]. Apart from its elastogenic function, fibulin-5 acts as an adhesion molecule via binding of its RGD motif to a number of integrins, including avb3, avb5, and a9b1 [16]. This reveals a critical role of fibulin-5 as a bridging molecule between cells and ECM.

Previous studies have identified fibulin-5 as a driving tumor suppressor in human cancers. Wen Yue et al. reported fibulin-5 suppresses lung cancer invasion by inhibiting MMP-7 expression [10]; Lee YH et al. found that fibulin-5 initiated and enhanced TGF-β-induced epithelial-mesenchymal transition (EMT) in mammary epithelial cells [17]; Zheng Hu et al. reported that down-regulation of fibulin-5 is related to the overexpression of VEGF and MYC in urothelial carcinomas of the bladder [9]. The role of fibulin-5 in tumor initiation and progression is still poorly understood. We hypothesize that the decreased expression of fibulin-5 disrupts the architecture of basement membranes and thereby enhances the ability of tumor cells to migrate and invade through the ECM. Moreover, fibulin-5 induced EMT, through which epithelial cells acquire phenotypes of motile fibroblasts, is a critical process in cancer invasion and metastasis [18].

CONCLUSION

In our study, we initially detected fibulin-5 expression status in 31 pairs of surgical resected CRC tissues. Our data indicated that the level of fibulin-5 expression in CRC was significantly lower than that in paired noncancerous tissues. Furthermore, fibulin-5 protein was expressed at significantly lower levels in CRC patients with lymph node metastasis and advanced TNM tumor stage. These results suggest that the reduced expression of fibulin-5 is correlated with poor prognostic features in CRC. However, more research needs to be done to discover the function of fibulin-5 in CRC. Altogether, we consider that fibulin-5 may potentially act as a diagnostic and/or prognostic biomarker, and may also be a possible target of preclinical gene therapy in CRC; however, this needs further investigation.

Support:
The authors received no funding for this article.

Declaration of Interest:
The authors declare that they have no competing interests.

References:

1. Stewart BW, Wild CP. World Cancer Report 2014. International Agency for Research on Cancer 2014;57-59 http://publications.iarc.fr/Non-Series-Publications/World-Cancer-Reports/World-Cancer-Report-2014.
2. Ó Céilleachair A, Hanly P, Skally M, et al. Counting the cost of cancer: out-of-pocket payments made by colorectal cancer survivors. Support Care Cancer 2017;1-9 (PMID: 28341973).
3. Yanagisawa H, Davis EC, Starcher BC, et al. Fibulin-5 is an elastin-binding protein essential for elastic fibre development *in vivo*. Nature 2002;415:168-71 (PMID: 11805834).
4. Timpl R, Sasaki T, Kostka G, Chu ML. Fibulins: a versatile family of extracellular matrix proteins. Nat Rev Mol Cell Biol 2003; 4:479-89 (PMID: 12778127).
5. Nakamura T, Ruiz-Lozano P, Lindner V, et al. DANCE, a novel secreted RGD protein expressed in developing, atherosclerotic, and balloon-injured arteries. J Biol Chem 1999;274:22476-83 (PMID: 10428823).
6. Heo JH, Song JY, Jeong JY, et al. Fibulin-5 is a tumour suppressor inhibiting cell migration and invasion in ovarian cancer. J Clin Pathol 2016;69:109-16 (PMID: 26251522).
7. Tu K, Dou C, Zheng X, et al. Fibulin-5 inhibits hepatocellular carcinoma cell migration and invasion by down-regulating matrix metalloproteinase-7 expression. BMC Cancer 2014;14:938 (PMID: 25494879).
8. Wlazlinski A, Engers R, Hoffmann MJ, et al. Downregulation of several fibulin genes in prostate cancer. Prostate 2007;67:1770-80 (PMID: 17929269).

9. Hu Z, Ai Q, Xu H, et al. Fibulin-5 is down-regulated in urothelial carcinoma of bladder and inhibits growth and invasion of human bladder cancer cell line 5637. Urol Oncol 2011;29:430-5 (PMID: 19767220).

10. Yue W, Sun Q, Landreneau R, et al. Fibulin-5 suppresses lung cancer invasion by inhibiting matrix metalloproteinase-7 expression. Cancer Res 2009;69:6339-46 (PMID: 19584278).

11. Schiemann WP, Blobe GC, Kalume DE, Pandey A, Lodish HF. Context-specific effects of fibulin-5 (DANCE/EVEC) on cell proliferation, motility, and invasion. Fibulin-5 is induced by transforming growth factor-beta and affects protein kinase cascades. J Biol Chem 2002;277:27367-77 (PMID: 12021267).

12. Shi XY, Wang L, Cao CH, Li ZY, Chen J, Li C. Effect of Fibulin-5 on cell proliferation and invasion in human gastric cancer patients. Asian Pac J Trop Med 2014;7:787-91 (PMID: 25129461).

13. Bissell MJ, Radisky D. Putting tumours in context. Nat Rev Cancer 2001;1:46-54 (PMID: 11900251).

14. Albig AR, Schiemann WP. Fibulin-5 antagonizes vascular endothelial growth factor (VEGF) signaling and angiogenic sprouting by endothelial cells. DNA Cell Biol 2004;23:367-79 (PMID: 15231070).

15. de Vega S, Iwamoto T, Yamada Y. Fibulins: multiple roles in matrix structures and tissue functions. Cell Mol Life Sci 2009; 66:1890-902 (PMID: 19189051).

16. Nakamura T, Lozano PR, Ikeda Y, et al. Fibulin-5/DANCE is essential for elastogenesis *in vivo*. Nature 2002;415:171-5 (PMID: 11805835).

17. Lee YH, Albig AR, Regner M, Schiemann BJ, Schiemann WP. Fibulin-5 initiates epithelial-mesenchymal transition (EMT) and enhances EMT induced by TGF-beta in mammary epithelial cells via a MMP-dependent mechanism. Carcinogenesis 2008;29: 2243-51 (PMID: 18713838).

18. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell 2009;139: 871-90 (PMID: 19945376).