# Fibulin-2 inhibits development of gastric cancer by downregulating β-catenin

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**Abstract.** The aim of the present study was to investigate the expression of fibulin-2 and β-catenin in gastric cancer tissues and the association to mutual regulation. Forty-nine cases of gastric cancer specimens obtained via surgical resection in the Pathology Department of Heping Hospital Affiliated to Changzhi Medical College from March 2013 to August 2017 were collected. The expression levels of fibulin-2 and β-catenin in 49 cases of gastric cancer and para-carcinoma tissues were detected via quantitative polymerase chain reaction and immunohistochemistry. The correlation of expression of fibulin-2 and β-catenin proteins in gastric cancer was detected via Spearman's analysis. The correlation of fibulin-2 and β-catenin protein expression with clinicopathological indexes of patients was also analyzed. Moreover, the fibulin-2 overexpression plasmid was constructed and transfected into human gastric cancer AGS and SGC-790 cell lines, so as to observe changes in β-catenin and its downstream indexes. Fibulin-2 messenger ribonucleic acid (mRNA) level in gastric cancer tissues was significantly lower than that in para-carcinoma tissues, while β-catenin mRNA level was significantly increased (P<0.05). The positive rate of fibulin-2 protein was 73.47% (36/49) in gastric cancer tissues and 16.33% (8/49) in para-carcinoma tissues, while that of β-catenin was 77.55% (38/49) in gastric cancer tissues and 28.57% (14/49) in para-carcinoma tissues, indicating that fibulin-2 protein is significantly deleted in gastric cancer tissues, and β-catenin protein is significantly upregulated (P<0.001). Fibulin-2 and β-catenin had a negative correlation (r=-0.361, P=0.003), but was closely correlated with the tumor size and lymph node metastasis (P<0.05). After overexpression of fibulin-2, expression of β-catenin, cyclin D1 and c-Myc protein was obviously downregulated (P<0.05). The tumor suppressor gene, fibulin-2, is significantly deleted in gastric cancer tissues, while β-catenin is remarkably enriched. Overexpression of fibulin-2 can inhibit the development of gastric cancer by downregulating β-catenin.

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## Introduction

Gastric cancer is the second most common cancer following lung cancer in China, as well as the second major cause of cancer death around the world (1). The onset of gastric cancer is considered as a gradual process caused by the abnormal activation of oncogene and inactivation of tumor suppressor gene (TSG). Once the oncogene is activated, carcinogenesis will be promoted by giving growth advantages. On the contrary, TSG is able to inhibit tumor growth and often inactivated during this process (2). The abnormal inactivation of TSG may be the result of combined effect of genetic and epigenetic changes. For example, hypermethylation of the promoter can lead to TSG transcriptional silencing, which is of great significance in tumorigenesis (3).

Fibulin is an extracellular matrix protein with multiple functions located on chromosome 3p21.1. It plays an important role in stabilizing the structure of basement membrane, elastic fibers and loose connective tissues and regulating the signal transduction between cells and between cells and matrix (4). Moreover, fibulin can also regulate cell morphology, growth, adhesion and movement. In human cancers, such as prostate cancer, some fibulin proteins are out of regulation (5), in which fibulin-1 and fibulin-2 are found to be downregulated in such cancers as prostate and breast cancer and play roles as TSG (6). However, the role of fibulin-2 in gastric cancer is still unclear.

β-catenin, a protein with dual function, has cell adhesion and signal transmission activity, was first discovered in the study on cell adhesion molecule E-cadherin (7). β-catenin is an indispensable part of the Wnt/β-catenin signaling pathway and plays a key role in the occurrence and development of gastric cancer (8). Previous studies have revealed that there is a direct or indirect regulatory relation between fibulin protein family and β-catenin, both of which exert an important influence on the development of gastric cancer (9).

The present study aims to further understand the roles of fibulin-2 and β-catenin in the pathogenesis of gastric cancer, and clarify whether there are significant differences in their expressions in gastric cancer and whether there is a correlation between them, so as to provide new ideas for the clinical diagnosis and treatment of gastric cancer.

## Materials and methods

*General data*. A total of 49 cases of gastric cancer specimens and corresponding para-carcinoma tissue specimens (>5 cm away from the edge of cancer tissues and pathologically confirmed as normal gastric mucosa) obtained via surgical resection in the Pathology Department of Heping Hospital Affiliated to Changzhi Medical College (Changzhi, China) from March 2013 to August 2017 were collected, fixed with 4% neutral formalin, routinely embedded into paraffin and serially sliced into 4 $\mu$m sections, followed by immunohistochemical labeling. After collection, the tissues were divided into 2 parts. One part of tissues was immediately placed into an Eppendorf (EP) tube containing ribonucleic acid (RNA) protective solution and then stored at -80˚C for subsequent experiments. The other part was added with tissue lysis solution to extract the total protein, and the protein expression level was detected via western blot analysis. The 49 gastric cancer patients were aged 34-87 years with an average age of 60 years, and there were 26 males and 23 females. In terms of tissue differentiation degree, there were 26 cases of high and moderate differentiation and 23 cases of low and no differentiation. In terms of tumor-node-metastasis (TNM) stage, there were 20 cases in stage I-II and 29 cases in stage III-IV. None of the patients underwent chemotherapy, radiotherapy and immunotherapy before the operation, and all of them were diagnosed via routine histopathology after operation.

This study was approved by the Ethics Committee of Heping Hospital Affiliated to Changzhi Medical College. Informed consent for the specimen collection was obtained from patients.

*Reverse transcription-polymerase chain reaction (RT-PCR)*. Gastric cancer and para-carcinoma tissues were ground with liquid nitrogen, and 1 ml TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was added to extract total RNA. The upper-layer liquid was taken, transferred into a 2.5 ml EP tube, placed on ice for 15 min and added with 200 $\mu$l chloroform, followed by centrifugation at 12,000 x g and 4˚C for 15 min. Then, the upper-layer liquid was taken carefully, transferred into a new EP tube, added with 200 $\mu$l isopropanol, vibrated gently several times and placed on ice for 10 min, followed by centrifugation at 12,000 x g and 4˚C for 15 min. After the supernatant was discarded, 2 ml 75% ethanol was added to wash RNA, followed by centrifugation at 12,000 x g and 4˚C for 10 min. The liquid was discarded and the RNA precipitate was dried at room temperature. Finally, an appropriate amount of RNase-free water was added to dissolve the RNA precipitate, and the RNA concentration was measured using a spectrophotometer. According to the experimental procedure provided by Takara (article no. 2690A), 1 $\mu$g RNA was taken for reverse transcription, and complementary deoxyribonucleic acid (cDNA) obtained was stored at -20˚C. The mRNA level of each index was detected according to instructions of the All-in-One™ qPCR Mix kit. The relative expression level of mRNA of each index was calculated as follows: $2^{-\Delta Cq}$ [$\Delta$Cq = Cq (target gene) - Cq (glyceraldehyde-3-phosphate dehydrogenase, GAPDH)] (10). The corresponding primer sequences are shown in Table I.

Table I. Primer sequences.

| Gene name | Primer sequences |
|---|---|
| *Fibulin-2* | 5'-GAGATCCCTGAGAGTGGCACTGAGG-3' |
| | 3'-GAGAAGGCACTCATCCTGGTCATCG-3' |
| *β-catenin* | 5'-CAAGGTGGGTGATGCCCTGAAGGAG-3' |
| | 3'-CGTCTGCACGGTCTTGAACTGGTCGTA-3' |
| *GAPDH* | 5'-AGGTCGGTGTGAACGGATTTG-3' |
| | 3'-GTAGACCATGTAGTTGAGGTCA-3' |

*Immunohistochemistry (IHC)*. After 49 cases of gastric cancer and para-carcinoma tissue specimens were fixed with 4% formaldehyde, routinely dehydrated and embedded into paraffin, the paraffin-embedded tissues were sliced into 4 $\mu$m sections, followed by IHC using streptavidin-peroxidase (SP) staining: After dewaxing with xylene and dehydration with gradient ethanol, antigen retrieval was performed using sodium citrate buffer via microwave, and the peroxidase was blocked with 3% $H_2O_2$ blocker. The sections were sealed with 10% donkey serum, phosphate-buffered saline (PBS) was added as the negative control, and the primary rabbit anti-human fibulin-2 and β-catenin polyclonal antibodies (cat. nos. ab251662 and ab16051, respectively; Abcam; diluted at 1:200) were added dropwise, followed by incubation in a wet box at 4˚C overnight. The next day, sections were washed with PBS 3 times, and incubated with the secondary goat anti-rabbit polyclonal antibody (cat. no. ab6721; Abcam; diluted at 1:200), followed by color development via diaminobenzidine (DAB) and photography under a microscope. The brown and dark brown nuclei under the microscope indicated positive cells, and the number of positive cells was counted. The number of positive cells/total number of cells in the visual field >5% indicated positive expression.

*Cell culture and plasmid transfection*. Human gastric cancer AGS and SGC-790 cell lines were purchased from the Chinese Academy of Sciences. The two kinds of cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare) and added with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin sodium and 100 $\mu$g/ml streptomycin (Hyclone; GE Healthcare). The cell culture flask was placed in an incubator containing 5% $CO_2$ at 37˚C.

Gastric cancer AGS and SGC-790 cells in the logarithmic growth phase were taken and digested with 0.25% trypsin. The cell density was adjusted, and cells were inoculated into a 24-well plate ($1x10^5$/well) and cultured for another 12 h, followed by transfection according to the instructions of Lipofectamine 2000. In the experiment, the cells were divided into the pcDNA-fibulin-2 group and control group. pcDNA-fibulin-2 (sequence: 5'-GTTAUUTACUCGTTGCGGUA-3', 5'-CUTTAGACUTTUGUGAGAUA-3') was produced by Shanghai GenePharma Co., Ltd.

*Western blot analysis*. After treatment, the cells were scraped off, transferred into a 2.5 ml EP tube and added with 150 $\mu$l

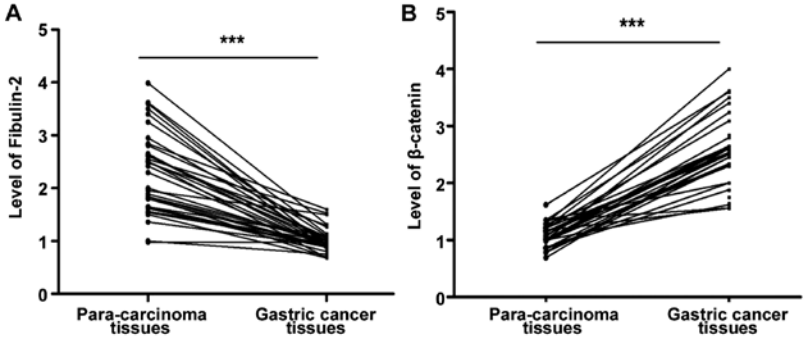


Figure 1. Expression of Fibulin-2 and β-catenin mRNA in gastric cancer and para-carcinoma tissues. (A) Fibulin-2 and (B) β-catenin mRNA expression in gastric cancer and para-carcinoma tissues. ***P<0.05 compared to para-carcinoma tissues.

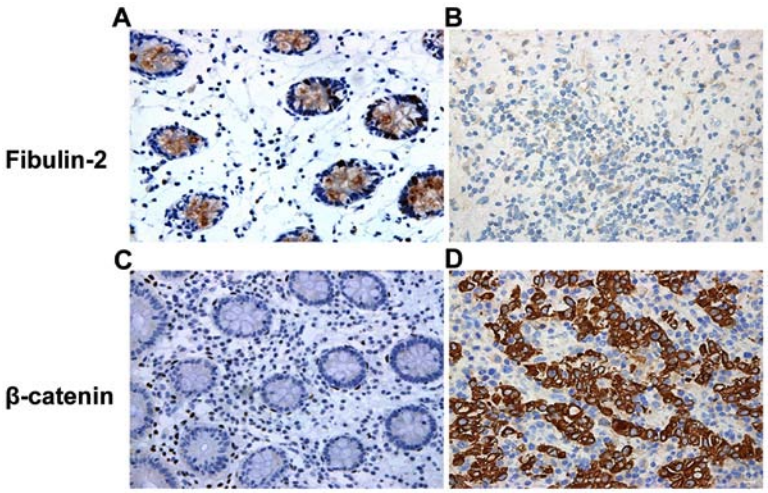


Figure 2. Expression of fibulin-2 and β-catenin protein in gastric cancer and para-carcinoma tissues (magnification, x400). (A) Fibulin-2 protein expression in para-carcinoma tissues. (B) β-catenin protein expression in gastric cancer. (C) Fibulin-2 protein expression in para-carcinoma tissues. (D β-catenin protein expression in gastric cancer.

mixture of radioimmunoprecipitation assay (RIPA) and protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA), followed by ultrasonic dispersion using an ultrasonic apparatus (40 A, 3 sec/time, repeated 3 times). After centrifugation at 12,000 x g at 4˚C for 15 min, the supernatant was taken as the tissue protein. The protein concentration was measured using the bicinchoninic acid (BCA) kit (Beyotime Institute of Biotechnoogy). After denaturation, the total protein was separated using 10% acrylamide gel, transferred onto a 0.22 $\mu$m nitrocellulose membrane (EMD Millipore) for 1.5 h, sealed with 5% skim milk for 1 h and incubated with rabbit anti-human fibulin-2, β-catenin, cyclin D1, C-myc and GAPDH polyclonal antibodies (cat. nos. ab251662, ab16051, ab226977, ab39688 and ab9485, respectively; Abcam; diluted at 1:1,000) overnight. Then the band was incubated with the goat anti-rabbit IgG secondary polyclonal antibody (cat. no. ab6721; Abcam; diluted at 1:300) for 1 h, and the target protein band was developed using the ECL system (Bio-Rad Laboratories). The relative content of the target protein was calculated as: gray value (target protein)/gray value (corresponding internal reference band).

*Statistical analysis*. GraphPad Prism 5.01 statistical software (GraphPad Software, Inc.) was used for the analysis of the experimental results. The independent-samples t-test was used for the comparison of difference in samples between two groups. Chi-square test was used for the comparison of difference in indexes between two groups. The correlation between fibulin-2 and β-catenin protein expression in gastric cancer was detected via Spearman's analysis. P<0.05 indicates the difference was statistically significant.

## Results

*Expression of Fibulin-2 and β-catenin detected via RT-PCR*. Fibulin-2 and β-catenin mRNA levels in gastric cancer and para-carcinoma tissues were quantitatively detected via RT-PCR. It was found that the fibulin-2 mRNA level in gastric cancer tissues was significantly lower than that in para-carcinoma tissues, while the β-catenin mRNA level in gastric cancer tissues was significantly higher than that in para-carcinoma tissues (P<0.05), and the mean differences were up to 2.25- and 3.02-fold higher, respectively (P<0.05; Fig. 1).

*Expression of Fibulin-2 and β-catenin protein in gastric cancer and para-carcinoma tissues detected via IHC*. As shown in Fig. 2, both fibulin-2 and β-catenin proteins were located in the cytoplasm, and the cytoplasm of positive cells were evident as brown yellow or dark brown color to

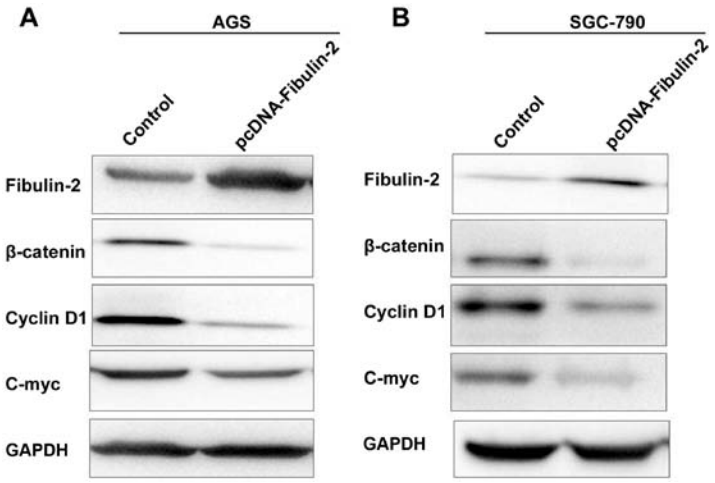


Figure 3. Overexpression of fibulin-2 significantly downregulates β-catenin. Protein expression in (A) AGS and (B) SGC-790 cell lines.

Table II. Expression of fibulin-2 and β-catenin protein in gastric cancer and para-carcinoma tissues.

| | | Fibulin-2 | | β-catenin | |
|---|---|---|---|---|---|
| Group | n | Negative n, (%) | Positive n, (%) | Negative n, (%) | Positive n, (%) |
| Gastric cancer tissues | 49 | 36 (73.47) | 13 (26.53) | 11 (22.45) | 38 (77.55) |
| Para-carcinoma tissues | 49 | 8 (16.33) | 41 (83.67) | 35 (71.43) | 14 (28.57) |
| $\chi^2$ | | 9.81 | | 6.84 | |
| P-value | | 0.001 | | 0.001 | |

varying degrees. The positive rate of fibulin-2 protein was 73.47% (36/49) in gastric cancer tissues and 16.33% (8/49) in para-carcinoma tissues, indicating that fibulin-2 protein is significantly deleted in gastric cancer tissues (P<0.001). The positive rate of β-catenin protein was 77.55% (38/49) in gastric cancer tissues and 28.57% (14/49) in para-carcinoma tissues, suggesting that β-catenin protein is significantly upregulated in gastric cancer tissues (P<0.001) (Table II).

*Correlation analysis between fibulin-2 and β-catenin*. The correlation between fibulin-2 and β-catenin protein expression in gastric cancer was analyzed via Spearman's correlation analysis, and it was found that they had a negative correlation (r=-0.361, P=0.003; Table III).

*Correlation of fibulin-2 and β-catenin expression with clinicopathological features of patients*. As shown in Table IV, the expression levels of fibulin-2 and β-catenin were not associated with the sex, age, differentiation degree, depth of infiltration and TNM stage of gastric cancer patients (P>0.05), but closely related to the tumor size and lymph node metastasis (P<0.05).

*Overexpression of fibulin-2 significantly downregulated β-catenin*. According to literature reports, fibulin-2 expression is low in AGS and SGC-790 cell lines. Therefore, fibulin-2 overexpression plasmid was transfected into the two cells. As shown in Fig. 3, after overexpression of fibulin-2, protein expression of β-catenin, cyclin D1 and c-Myc was obviously downregulated (P<0.05).

Table III. Correlation between fibulin-2 and β-catenin in gastric cancer tissues.

| | Fibulin-2 | | | |
|---|---|---|---|---|
| Protein expression | Negative (36) | Positive (13) | r | P-value |
| β-catenin | | | | |
| Negative (11) | 6 | 10 | -0.361 | 0.003 |
| Positive (38) | 27 | 6 | | |

## Discussion

Previous findings revealed that fibulin-2 is one of the 64 genes overexpressed in solid tumors from different sources (11). However, fibulin-2 silencing was found in studies on colorectal cancer (23 cases), liver cancer (24 cases), prostate cancer (25 cases) and nasopharyngeal cancer (26 cases) (12-14). According to recent studies, type I transmembrane glycoprotein whose expression is enhanced in pancreatic cancer interacts with fibulin-2 via the nestin-like domain, and fibulin-2 is one of the related genes promoting pancreatic cancer MC-4 cell-mediated metastasis (15). The proteomic analysis of the mouse model of tumor showed that fibulin-2 may be a tissue and plasma biomarker for breast cancer. In addition, several studies have demonstrated that the inactivation of fibulin-2 is correlated with the tumor progression (16,17). The 10K array

Table IV. Association of fibulin-2 and β-catenin expression with clinicopathological characteristics of patients.

| | | Fibulin-2 | | | β-catenin | | | |
|---|---|---|---|---|---|---|---|---|
| Index | n | Negative (13) | Positive (36) | P-value | n | Positive (38) | Negative (11) | P-value |
| Sex | | | | 0.245 | | | | 0.315 |
| Male | 26 | 6 | 20 | | 27 | 21 | 6 | |
| Female | 23 | 7 | 16 | | 22 | 17 | 5 | |
| Age (years) | | | | 0.135 | | | | 0.262 |
| ≤60 | 24 | 8 | 16 | | 23 | 18 | 5 | |
| >60 | 25 | 5 | 20 | | 26 | 20 | 6 | |
| Tumor size (cm) | | | | 0.038 | | | | 0.019 |
| ≤3 | 16 | 4 | 12 | | 19 | 10 | 9 | |
| >3 | 33 | 9 | 24 | | 30 | 28 | 2 | |
| Differentiation degree | | | | 0.224 | | | | 0.092 |
| High and moderate differentiation | 26 | 6 | 20 | | 20 | 13 | 7 | |
| Low and no differentiation | 23 | 7 | 16 | | 29 | 25 | 4 | |
| Depth of infiltration | | | | 0.261 | | | | 0.085 |
| T1-T2 | 22 | 5 | 17 | | 21 | 17 | 4 | |
| T3-T4 | 27 | 8 | 19 | | 28 | 21 | 7 | |
| Lymph node metastasis | | | | 0.041 | | | | 0.023 |
| No | 19 | 8 | 11 | | 20 | 11 | 9 | |
| Yes | 30 | 5 | 25 | | 29 | 27 | 2 | |
| TNM stage | | | | 0.108 | | | | 0.081 |
| I-II | 20 | 9 | 11 | | 22 | 17 | 5 | |
| III-IV | 29 | 4 | 25 | | 27 | 21 | 6 | |

analysis of human genomic map revealed that the chromosome 3p25.1 region (fibulin-2) in patients with esophageal squamous cell carcinoma is often deleted, indicating that fibulin-2 is a potential TSG (18). Studies on liver cancer have suggested that the anti-angiogenic effect of fibulin-2 is accompanied by the downregulation of two pro-angiogenic factors, vascular endothelial growth factor and matrix metalloproteinase-2 (19). According to subsequent epigenetic research, the inactivation of fibulin-2 in a variety of tumors is caused by the abnormal increase of methylation level (20). However, neither expression nor function of fibulin-2 in gastric cancer has been clear as yet.

The activation of Wnt/β-catenin pathway is a key carcinogenic event in the occurrence, development and metastasis of tumors. β-catenin is a kind of oncoprotein generally located in the cytoplasm, whose expression is inhibited by ubiquitin/proteasome-mediated protein degradation (21). In adenomatous polyp, the glycogen synthase kinase 3β (GSK3β)-mediated carcinogenic Wnt signal promotes the accumulation and nuclear translocation of glycogen synthase kinase 3β (GSK3β)-catenin, thus forming the complex. Factors such as T-cell factor 4 (TCF-4) lead to the activation of downstream targets, such as oncogenes C-myc and cyclin D1, and the Wnt/β-catenin signal transduction is often activated in tumors, thereby promoting the proliferation, survival and metastasis of tumor cells (22,23).

In this study, 48 cases of gastric cancer were collected. Both mRNA and protein levels of fibulin-2 in gastric cancer tissues were significantly downregulated, while those of β-catenin were significantly upregulated. The positive rate of fibulin-2 protein was 73.47% in gastric cancer tissues and 16.33% in para-carcinoma tissues, while positive rate of β-catenin protein was 77.55% in gastric cancer tissues and 28.57% in para-carcinoma tissues. Then the correlation between expression of fibulin-2 and β-catenin protein in gastric cancer was analyzed via Spearman's correlation analysis, and it was found that they were negatively correlated (r=-0.361, P=0.003). Furthermore, gastric cancer AGS and SGC-790 cell lines were selected and transfected with fibulin-2 overexpression plasmid, and β-catenin and its downstream regulatory molecules (C-myc and cyclin D1) were detected via western blot analysis. Results showed that β-catenin and its downstream molecules were also inhibited, which is consistent with findings obtained in clinical tissue specimens. According to previous findings, fibulin-2 has the largest molecular weight in the fibulin family, which can interact with various cytoplasmic proteins. Such interactions may be critical in cell movement and proliferation (24). It is hypothesized that the downregulation of fibulin-2 promotes the proliferation of gastric cancer through the abnormal activation of β-catenin, and the β-catenin signal also plays an important role in gastric cancer. The constantly high expression level of β-catenin in gastric cancer cells leads to the activation of TCF-4 and the overexpression of cyclin D1, thus promoting the proliferation of gastric cancer cells. The specific molecular mechanisms remain unclear, but the expression has correlation in tumors with larger volume and deeper infiltration,

further confirming the role of fibulin-2 in the mechanism of proliferation of gastric cancer. The above results indicate that the *in vitro* and *in vivo* functional characteristics of fibulin-2 reveal the important antitumor effect of fibulin-2 in cell proliferation, migration and invasion, which provides the first clue for the functional and molecular characteristics of fibulin-2 in tumor progression.

In conclusion, results of the present study indicate that fibulin-2 plays an important role in inhibiting gastric cancer invasion, which is mediated by the inhibition of β-catenin signal. Further research on this pathway may provide potentially useful information for the development of biological or pharmacological drugs for metastatic lung cancer.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

HM and CL performed the immunohistochemistry and PCR. HM and YS were responsible for the cell culture and western blotting. HM wrote the manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The present study was approved by the Ethics Committee of Heping Hospital Affiliated to Changzhi Medical College (Changzhi, China) and informed consents were signed by the patients or their guardians.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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