RESEARCH ARTICLE

Interactions between Filamin A and MMP-9 Regulate Proliferation and Invasion in Renal Cell Carcinoma

Guo-Gui Sun¹, Cui-Da Wei², Shao-Wu Jing³, Wan-Ning Hu¹*

Abstract

This study aimed to analyze the expression, clinical significance of filamin A (FLNA) in renal cell carcinoma (RCC) and biological effects in a cell line by regulating FLNA expression. Immunohistochemistry and Western blotting were used to analyze FLNA protein expression in 70 cases of RCC and normal tissues to study the relationship with clinical factors. FLNA lentiviral and empty vectors were transfected into RCC to study the influence of up-regulated expression of FLNA. FLNA siRNA was transiently transfected into ACHN kidney carcinoma cells by a liposome-mediated method and protein was detected by Western blotting. The level of expression was found to be significantly lower in RCC than normal tissues (p<0.05). No correlation was noted with gender, age, tumor size or pathological types (p>0.05), but links with lymph node metastasis, clinic stage and histological grade were noted (p<0.05). Loss of FLNA expression correlated significantly with poor overall survival time by Kaplan-Meier analysis (p<0.05). Results for biological function showed that ACHN cells transfected with FLNA had a lower survival fraction, significant decrease in migration and invasion, higher cell apoptosis, higher percentage of the G0/G1 phases, and lower MMP-9 protein expression compared with ACHN cells untransfected with FLNA (p<0.05). However, renal 786-0 cells transfected with FLNA siRNA had a higher survival fraction, significant increase in migration and invasion, and higher MMP-9 protein expression compared (p<0.05). In conclusion, FLNA expression was decreased in RCC and correlated significantly with lymph node metastasis, clinic stage, histological grade and poor overall survival, suggesting that FLNA may play important roles as a tumor suppressor in RCC by promoting degradation of MMP-9.

Keywords: FLNA - renal cell carcinoma - MMP-9 - immunohistochemical analysis

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Introduction

The incidence of renal cell carcinoma (RCC) varies substantially worldwide. The rates are generally high in Europe and North America while low in Asia and South America (Ljungberg et al., 2011). Metastatic RCC is a highly fatal disease, which accounts for about a third of the patients at initial presentation. Approximately 10% to 28% of RCC develop a local recurrence or distant metastasis after curative nephrectomy. Metastatic RCC is resistant to chemotherapy and radiotherapy but responds to tyrosine kinase inhibitors and interleukin-2-based immunotherapy (Tan et al., 2013). RCC is heterogeneous and comprises several histological cell types with different genetics, biology and behavior. The identification of the genes predisposing to inherited syndromes with RCC has provided much of our knowledge of the molecular basis of early sporadic RCC. Many of the oncogenes and tumor suppressor genes that are mutated leading to pathway dysregulation in RCC remain to be elucidated.

Global studies of copy number, gene sequencing, gene expression, miRNA expression and gene methylation in primary RCC will lead towards this goal (Stadler et al., 2010).

Although significant progression has been made in both basic and clinical research in RCC, the molecular mechanism responsible for development of RCC, like most other cancers, remains to be determined, and a number of gene alterations may contribute to renal cell carcinogenesis (Stadler et al., 2010). Thus, further studies will clarify the pathogenesis of RCC and provide the molecular targets for effective treatment of RCC. FLNA, a 280-kDa actin-binding protein encoded by an X-linked gene, has been demonstrated to interact with more than 70 diverse cellular proteins, including signaling molecules and membrane receptors. It thus provides essential scaffolding functions and crucial links from receptor-mediated signal transduction to the actin cytoskeleton (Stossel et al., 2001; Nakamura et al., 2007; Zhou et al., 2007; Ai et al., 2011). It has been reported that FLNA
is required for the migration of cortical neurons and melanoma cells and metastatic progression of prostate cancer and ovarian cancer (Stossel et al., 2001; Sheen et al., 2002; Kenwrick et al., 2003; Feng et al., 2004; Keshamouni et al., 2006; Bourguignon et al., 2007; Bedolla et al., 2009). Hence, a role for FLNA in RCC tumorigenesis and metastasis is to be expected.

Materials and Methods

Main Reagents

Rabbit anti-human FLNA monoclonal antibody (Amcam Inc., USA). Rabbit anti-human MMP-9 monoclonal antibody (Amcam Inc., USA). Bovine serum albumin (BSA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primary antibody were purchased from Sigma Chemical Company (St. Louis, MO, USA). Plenti6/V5-DEST Vector, lentiviral packaging mix, Opti-MEM, Lipofectamine 2000, and the SuperScript III Reverse Transcriptase (RT) kit were obtained from Invitrogen Corporation (Carlsbad, CA, USA). TaqDNA polymerase was purchased from Fermentas, Inc. (Waltham, MA, USA). The DyLight Fluor conjugated to goat anti-rabbit IgG was obtained from LI-COR bioscience, Inc. (Lincoln, Nebraska, USA). An immunohistochemistry kit was purchased from 4A Biotech Co. Ltd. (Beijing, China). Fetal bovine serum (FBS), cell-culture media, and supplementary materials were obtained from Gibco Co. (Grand Island, NY, USA).

Clinical Data

There were 48 males and 22 females, aged from 21 to 73 years old, with a median age of 53 years. Of the 70 cases of RCC: 25 of them with lesion ≤7 cm and 45 with lesion > 7cm. The included studies contained 43 cases of clear cell type, 18 cases of granule cell type, and 9 cases of papillary cell type. Meanwhile, 27 patients demonstrated no lymph node metastasis (NO), whereas 43 with identified lymph nodes involvement (N+). As for the clinic stages, 23 cases had I-II stages and 47 had a III-IV stages. The grades of differentiation were 29 with Grade I (well differentiated) and 41 with Grade II or III (moderately to poorly differentiated). All of the patients did not have any no adjuvant therapies. The fresh specimens of tumor tissue without knowing patients' information were responsible for each specimen were counted. FLNA expression was classified as: 0 point: no staining, 1 point: weak staining (light yellow); 2 points: moderate staining (brown); and 3 points: strong staining (yellowish-brown). The final score of FLNA expression was the product of the FLNA expression rate and intensity, graded as 0 for negative, + for 1-3 points, ++ for 4-6 points, and +++ for 7-9 points. As for the negative control, the primary antibody was replaced with PBS.

Immunohistochemistry

A 4 μm section was prepared from paraffin-embedded block and dehydrated, then incubated in 3% hydrogen peroxide for 10 min to block endogenous peroxidase, followed by using trypsin for repair of 20 min; 10% goat serum was introduced at room temperature for closure of 20 min, and FLNA antibody (1: 100) was left in the wet box at 4°C refrigerator for overnight. Then the secondary and third antibodies were dropped into the wet box at room temperature for incubation of 20 min, respectively; DAB staining was visualized by the hematoxylin stain, and then came to normal dehydration with the coverslip sealed. Results evaluation: two pathologists for each specimen were counted. FLNA expression was determined based on the percentage of positive cells, combined with the staining intensity. The percentage of positive cells was divided into four levels: 0 point: ≤5% of positive cells, 1 point: 5%~25%, 2 points: 25%~50%, and 3 points: >50% of positive cells. The intensity of staining was classified as: 0 point: no staining, 1 point: weak staining (light yellow); 2 points: moderate staining (brown); and 3 points: strong staining (yellowish-brown). The final score of FLNA expression was the product of the FLNA expression rate and intensity, graded as 0 for negative, + for 1-3 points, ++ for 4-6 points, and +++ for 7-9 points.
Semi-quantitative RT-PCR

Total RNA extraction: specimen was removed from the liquid nitrogen, and the total RNA was extracted according to the instructions on Trizol reagent. 2 μg total RNA was taken for synthesis of cDNA according to the operating requirements of Superscript III Reverse Transcriptase Kit Instructions in 20 μl reaction system; Reaction conditions: denaturation at 95℃ for 5 min, and RT at 50℃ for 50 min. PCR amplification of FLNA gene upstream primer: 5’-AGGCCCTCAGGACATCATC-3’, downstream primer: 5’-CCAGTGTGACTTCCCCCTTG-3’. GAPDH gene upstream primer: 5’-CCGACCTGGCCCTTCACTAGA-3’, downstream primer: 5’-CTGGGGCTTAACATCTCC-3’. PCR reaction 50 μl include: 5 μl 10× PCR buffer, 1 μl 10 mmol dNTP, 0.5 μl TaqDNA polymerase, the upstream and downstream primers 2 μl, respectively, 2 μl template cDNA, plus ddH2O, was complemented to 50 μl. Reaction conditions: pre-denaturation at 95℃ for 3 min, denaturation at 95℃ for 30 s, annealing at 56℃ for 30 s, extension at 72℃ for 45 s, a total of 35 cycles, placed at -20℃. PCR product detection and its semi-quantitative analysis: after the product underwent gel electrophoresis in 1.5% agarose, the gel imaging system combined with Multi Gauge V 3.1 was used for optical density analysis of the results.

Western blot

All operations were completed on the ice. At 4℃, 12000 r/min centrifugation lasted for 20 min, and then the supernatant was taken for backup at -20℃. After the detection of protein concentration with BCA Protein Assay Kit, each hole was given a sample amount of 50 μg for SDS-PAGE electrophoresis. Regulator power for ice bath was transferred to nitrocellulose membrane, followed by closed by incubation for 2 h with 5% skim milk. Subsequent to Anti-1 overnight incubation at 4℃ (FLNA 1:1000, MMP-9 1:1000, GAPDH 1:3000). The latter was from Sigma Chemical Company, St. Louis, MO, USA) in 5% nonfat dry milk for 1 h at room temperature. After washing, the membrane was incubated with goat anti-rabbit fluorescent secondary antibody (IRDye800, 1:20, 000 dilution; the DyLight Fluor conjugated to goat anti-rabbit IgG was obtained from LI-COR Bioscience, Inc., Lincoln, Nebraska, USA) in the dark for 1 h at room temperature. The blots were then scanned and analyzed using the Odyssey Infrared Imaging System (LI-COR Bioscience). Western blot data were quantified by normalizing the signal intensity of each sample to that of GAPDH.

MTT assay

Cell viability was determined by using the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were plated into 96-well culture plates at an optimal density of 5×10^4 cells/ml in 200 μl of culture medium per well. After 24-96 h of culture, 20 μl of 5 mg/ml MTT was added to each well and incubated at 37℃ for 4 h. The medium was then gently aspirated and 150 μl of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals. The optical density of each sample was immediately measured using a microplate reader (BioRad) at 570 nm.

Flow cytometry assay

Cell apoptosis: An Annexin V-FITC-flow cytometry assay was used to detect the apoptosis rate in the cells after FLNA transfection. Cells were seeded into 60-mm dishes for 48 h and grown to approximately 70-75% confluence. After quick detachment from the plate, cells were collected, washed with ice-cold PBS, and resuspended at a cell density of 1×10^6/ml in a binding buffer from the Annexin V-FITC apoptosis detection kit (4A Biotech Co. Ltd, Beijing, China) and then stained with 5 μl of Annexin V-FITC and 10 μl of propidium iodide (PI, 20 μg/ml). The cells were then incubated in the dark at 25℃ for 15 min before 10,000 cells were analyzed by a FACSScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA) and CellQuest software (Becton-Dickinson) for apoptosis rate determination. The data were evaluated using the ModFit software program (Verity Software House, Topsham, ME, USA). Cell cycles: For cell cycle distribution, PI staining and the flow cytometry assay were performed as described previously. Approximately 1×10^4 cells were fixed in 70% ethanol and resuspended in 1 ml of a solution containing 3.8 mM sodium citrate, 50 μg/ml propidium iodide, and 0.5 μg of RNase A, and analyzed with a flow cytometer. The data were evaluated using the ModFit software program.

Invasion and migration assays

The Costar Transwell 8 μm inserts were coated with 50 μg reduced serum Matrigel (BD Biosciences Franklin Lakes, NJ) for invasion assay according to the manufacturer’s instruction. Invasion Chambers (BD China, Shanghai, China) at 1×10^5 cells per chamber. The membrane in the chamber was coated with Matrigel (BD China). Medium supplemented with 10% FBS was used in the lower chamber. Migration assays were performed in the same manner excluding the Matrigel. After 12 h, non-invading cells, and media were removed with a cotton swab. Cells on the lower surface of the membrane were fixed with polyoxyethylene (Sigma) and stained with 0.1% crystal violet (Sigma) for 0.5 hours. Stained cells were counted under a microscope in 4 randomly selected fields, and the average was used to indicate cell migration and invasion.

Statistical analyses

All statistical analyses were performed using SPSS16.0 software. For the clinicopathologic features, P values were calculated using the χ^2 test. FLNA mRNA or protein expression was shown in mean±SD. Student t-test was used to analyze the difference between two groups. A 5% or lower P-value was considered to be statistically significant.

Results

FLNA protein expression in RCC and normal tissue

In RCC tissues, FLNA staining was negative or weak. In normal renal tissues, FLNA staining ranged from light yellow to brown. Statistically, FLNA was expressed in 37.1% (26/70) of RCC tissues, which was lower than the 71.1% (27/38) in normal tissues. The difference was
statistically significant ($p<0.05$) (Table 1, Figure 1). FLNA protein expression level in renal cell cancer was 0.269±0.035, lower than the 0.832±0.075 expression in normal tissue, indicating a difference with statistical significance ($p<0.05$) (Figure 2). The expression of FLNA was correlated with clinical stages, lymph node metastasis and pathological differentiation ($p<0.05$), regardless of age, gender, tumor size and pathological types ($p>0.05$) (Table 2).

**FLNA expression and prognosis**

Survival analysis was performed in all the patients and follow-up data were collected. All patient follow-ups ended in 2013 after a revisit time of 60 months. Among all cases, 30 were still alive at this time and 40 were dead. Patients were divided into two groups according to FLNA expression level. There were 26 individuals with positive levels of FLNA expression, among whom 16 were still alive and 10 were dead. The survival rate was 61.5%. There were 44 individuals with negative levels of FLNA expression, among whom 14 were still alive and 30 were dead. The survival rate was 31.8%. Patients with high levels of FLNA expression had significantly higher 5-year survival rates than those with low levels of FLNA expression group ($p<0.05$).

**Stable transfection of FLNA cDNA in RCC cell and on effect of RCC cell**

In this study, we first stably transfected FLNA cDNA into ACHN cell and obtained overexpressed FLNA ACHN sublines (named as LeFLNA cell) and empty vector-transfected cell (named as LeEmpty cell) as the control. RT-PCR data showed that the FLNA mRNA expression level was 0.424±0.039 in empty vector-transfected cells. In contrast, the amount of FLNA mRNA in the FLNA-transfected cell lines was 1.153±0.102. The difference was statistically significant ($p<0.05$, Figure 3A). Furthermore, western blot analysis showed that the control cells had approximately equal amounts of immunoreactive protein (0.434±0.041). In contrast, the amount of FLNA protein in the FLNA-transfected cell lines was 0.945±0.113. The difference was statistically significant ($p<0.05$, Figure 3B). Next, we assessed the effect of FLNA expression on the regulation of ACHN cell viability. MTT assay showed that relative proliferative capacity of the LeFLNA cell relative grew slower at 24, 48, 72 h and 96 h compared with the parental LeEmpty cell. The difference was statistically significant ($p<0.05$, Figure 3C). Cell cycle analysis using flow cytometry showed that the proportion of Le FLNA cells in G0/G1 and S phases of the cell cycle were significantly different compared to the control LeEmpty cells (74.9±5.3 and 8.8±0.7%, vs 44.3±3.1 and 34.5±2.6%, respectively; $p<0.05$, Figure 3D). In addition, there was a large increase in the early and late apoptosis rate in Le FLNA cells compared to control

Table 1. Expressions of FLNA in RCC Tissue and in Normal Renal Tissue

<table>
<thead>
<tr>
<th>Groups</th>
<th>Case</th>
<th>Expression of FLNa Protein</th>
<th>–</th>
<th>+</th>
<th>++</th>
<th>+++</th>
<th>χ²</th>
<th>p</th>
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Table 2. Relation between FLNA Expression and Clinic Characteristics in RCC

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<th>+~+++</th>
<th>χ²</th>
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<td>Papillary cell type</td>
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Figure 1. Expressions of FLNA Protein in RCC and Normal Tissue. A and B RCC. A) SP×100, B) SP×400; C and D normal tissue. C) SP×100, D) SP×400

Figure 2. Expressions of FLNA Protein in RCC and Normal Tissue. A) RCC; B) normal tissue. *$p<0.05$ compared to the normal tissue
Figure 3. Expression Identification of the FLNA Gene and The Effects of FLNA Overexpression on RCC ACHN cell. A) RT-PCR, 1. LeEmpty Cell; 2. LeFLNA cell; B) Western blot, 1. LeFLNA cell, 2. LeEmpty cell; C) Cell proliferation; D) Cell cycles; E) Migration and invasion; F) Western blot. *p<0.05 compared to the LeEmpty cell

Figure 4. Expression identification of the FLNA siRNA and The effects of FLNA siRNA on RCC 786-0 cell. A) Western blot, 1. FLNA siRNA cell, 2. Control cell; B) Cell proliferation; C) Migration and invasion; D) Western blot, 1. Control cell, 2. FLNA siRNA cell. *p<0.05 compared to the Control cell
LeEmpty cells (18.9±2.1 vs 7.4±1.1%; p<0.05) (Figure 3E). Furthermore, the renal cell cancer cell ACHN cells transfected with FLNA or empty vector were transferred to transwell chambers or Matrigel-coated transwell chambers to evaluate the effect of FLNA on cell migration and invasion potential. Introduction of FLNA clearly led to a significant decrease in ACHN cell migration and invasion (58.0±6.0 and 43.0±4.0, respectively) compared with the cells transfected with empty vector (82.0±7.0 and 64.0±5.0, respectively). The difference was statistically significant (p<0.05, Figure 3F).

To further identify the mechanisms by which FLNA inhibited renal cell cancer cell proliferation, migration, and invasion, we analyzed the expression level of MMP-9 due to their critical roles in cell proliferation and invasion. Western blot analysis revealed that FLNA significantly reduced MMP-9 protein expression in LeFLNA cell (0.359±0.041) compared to controls cells (0.843±0.087), indicating a difference with statistical significance (p<0.05, Figure 3G).

Effects of FLNA siRNA on RCC 786-0 cell
FLNA siRNA was transiently transfected into kidney carcinoma 786-0 cell by liposome-mediated method and was detected by western blot. Western blot data showed that the FLNA protein expression level was 0.985±0.113 in empty vector-transfected cells. In contrast, the amount of FLNA protein in the FLNA siRNA -transfected cell lines was 0.435±0.041. The difference was statistically significant (p<0.05, Figure 4A).

Furthermore, we assessed the effect of FLNA siRNA on the regulation of kidney cancer cell viability. MTT assay showed that relative proliferative capacity of the FLNA siRNA cell relative grew higher at 24, 48, 72 h and 96 h compared with the parental cell. The difference was statistically significant (Figure 4B, p<0.05). Meanwhile, the 786-0 cells transfected with FLNA siRNA or empty vector were transferred to transwell chambers or Matrigel-coated transwell chambers to evaluate the effect of FLNA siRNA on cell invasion potential. Introduction of FLNA siRNA clearly led to a significant increase in 786-0 cell migration and invasion (206.0±26.0 and 93.0±14.0, respectively) compared with the cells transfected with empty vector (135.0±15.0 and 65.0±7.0, respectively). The difference was statistically significant (p<0.05, Figure 4C).

Western blot analysis revealed that FLNA significantly increased MMP-9 protein expression in FLNA siRNA cell (0.963±0.097) compared to controls cells (0.433±0.041), indicating a difference with statistical significance (p<0.05, Figure 4D).

Discussion
Using immunohistochemistry and Western blot techniques, our study for the first time compared the distribution and expression of FLNA in RCC tissues and in normal renal tissues. Our results showed the downregulation of protein expressions of FLNA in RCC tissues as compared with normal renal tissues. Univariate analysis revealed that the protein expressions of FLNA were correlated with the lymph node metastasis, clinical stage and tumor differentiation, but were not associated with the gender, age, tumor size and of patients with RCC. Furthermore, survival analysis showed that the five-year overall survival rate for patients with FLNA positive expression was greatly higher than those FLNA-negative patients. These findings are in accordance with previous report which shows the increased FLNA-positive expression in healthy rectal tissues compared to colorectal adenocarcinoma tissues (Shi et al., 2010). Contrast results are obtained in invasive breast cancer, as the level of FLNa is upregulated with increased tumor differentiation (Wu et al., 2010). Using immunohistochemistry, Bedolla et al. analyzed the expression of FLNA in samples obtained from patients with different stages of prostatic cancer and found that the cytoplasmic level of FLNA was obviously downregulated in metastatic prostate cancer as compared with benign prostatic samples, prostatic intraepithelial neopvlasia, or prostate cancer is situ, whereas the nuclear expression of FLNA was significantly elevated in metastatic prostate cancer, which might be due to enhanced grade of malignancy (Feng et al., 2004). Such discrepancy might be due to the varied regulatory role of FLNa on different types of tumor cells. The underlying mechanism still needs to be further clarified.

The invasive growth is a critical property of malignant cancer. It is also the main reason for the failure of therapy for cancer patients, and may even leads to death. Degradation of extracellular matrix is believed to be the prediction for the initiation of tumor cell invasion. Matrix metalloproteinase (MMP) is a family of highly conserved proteins, which are synthesized and secreted by endothelial cells, granulocytes and tumor cells (Watanabe et al., 2010). MMP-9 is one of the MMP family members which has the largest relative molecular mass. The substrate for MMP-9 is gelatin, type IV, and type V collagen. Upregulated expression of MMP-9 is detected in various types of malignant tumors, cultured tumor cells and cells transfected with tumor genes. In vitro tumor invasion experiments shows that the enhanced capability of tumor cell invasion is associated with elevated MMP-9 expression (Yu et al., 2011; Zhang et al., 2011). In addition, in vitro study for the first time showed that the renal cell carcinoma cells with upregulated FLNA expression, exhibited significantly decreased invasive and metastatic capabilities, and the MMP-9 level was also reduced in these cells. Further in vitro experiments demonstrated that kidney cancer cells with high FLNA siRNA had significantly increased proliferation and migration and invasion, and markedly increased MMP-9 protein levels. Melanoma M2 cells lack the FilaminA expression, while the subclone M2A7 cells can stably express the FLNA protein. It is reported that the secretion capability of MMP-9 is greatly enhanced in M2 cells as compared with M2A7 cells, as FilaminA can regulate the ERK activity and downregulate MMP-9 expression through suppressing Ras/MAPK signals (Yang S et al., 2007). MAPK/ERK signal pathway regulates several critical pathophysiological processes, including cell growth, division, differentiation, apoptosis, adaption to environment, response to inflammation. By interacting with the upstream molecules, FLNA mediates the activity of MAPK/ERK signal pathway. FLNA binds to the opioid
Filamin A and MMP-9 Regulate Proliferation and Invasion in Renal Cell Cancer


