Filamin A regulates EGFR/ERK/Akt signaling and affects colorectal cancer cell growth and migration

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Abstract. The metastasis and recurrence rate, and the overall prognosis of colorectal cancer (CRC) remain unsatisfactory. Filamin A (FLNa), as an actin-binding protein, can interact with various signaling molecules and membrane receptors to affect cell signal transduction and function. However, whether FLNa is involved in the progression of CRC remains to be elucidated. The aim of the present study was to explore the role of FLNa in CRC cell proliferation and migration, as well as in the regulation of epidermal growth factor receptor (EGFR) signaling. Following transfection with a FLNa-targeting short hairpin RNA plasmid to knockdown expression of FLNa in the EGF-treated SW480 cell line, it was found that decreased expression of FLNa promoted cell proliferation and migration. Additionally, there was a negative correlation between FLNa levels and the activation of EGFR and Akt signaling pathways. Similarly, the expression of FLNa was significantly lower in human CRC tissues compared with adjacent normal tissues and FLNa expression was negatively correlated with the expression of Ki-67 in human CRC tissues. Although there was no significant difference in the Kaplan-Meier estimate of CRC between high expression and low expression of FLNa, there were significant negative associations between FLNa expression and TNM stage. The results suggested that FLNa may participate in EGFR-induced cell proliferation and migration in CRC cells. Hence, interventions in the FLNa-mediated signaling pathway could provide attractive therapeutic targets for CRC.

Introduction

Colorectal cancer (CRC) is the third-leading cause of cancer mortality in most countries, and there were an estimated 134,490 new cases and 49,190 deaths in the United States in 2015 (1,2). Although the mortality rate from CRC has declined by 3% over the past decade, 30-40% of CRC patients still develop metastases and 50% die of CRC recurrence (3). CRC, which is associated with poor prognosis, is usually diagnosed at a late stage (4-7). Critical cancer hallmarks include the ability of tumor cells to invade and migrate to distant tissues and to exhibit enhanced cell proliferation (8); therefore, elucidation of these cancer-promoting mechanisms may lead to drug discoveries for novel therapies and subsequent improvement of survival rates of CRC patients.

Filamins (FLNs), a family of actin-binding proteins, comprise FLNa, FLNb and FLNc. FLNa, also known as filamin-A, was identified in macrophages in 1975 and was originally characterized as a protein that could cross-link actin filaments and form rigid gels (9-11). Its contribution to the formation of the actin cytoskeleton serves a vital role in responses to extracellular stimulation. The molecular function of FLNa in cell chemotaxis remains debatable and seems to vary according to the levels of FLNa and its interacting partners (12). Abnormal expression of FLNa is associated with a wide spectrum of human disorders (13-17) that are caused by abnormal interactions between FLNa and its corresponding partners (18,19). Zhu et al (20)

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SW480 cells were seeded at a density of 4x10^5/ml, cultured in serum-free 1640 medium (SFM) for 2 h, the cells were treated with 600 µl of RPMI 1640/10% FBS medium. Cells were allowed to migrate for 24 h (untreated or treated with 20 nM EGF). Non-migratory cells on the upper surface were then removed using a wet cotton swab. The migrated cells on the surface of the lower membrane were stained by H&E and counted under a light microscope (magnification x200; Olympus Corporation) in 5 random fields to obtain the average number.

Western blotting. SW480 cells were treated with or without EGF (20 nM) for 5, 10 and 30 min and then lysed with cold lysis buffer (Amresco Scientific, Inc.). The protein concentration was quantified using a bicinchoninic acid assay kit. Proteins (20 µg/ lane) were separated via 10% SDS-PAGE and were transferred onto polyvinylidene difluoride membranes. Subsequently, PVDF membranes were blocked with 3% bovine serum albumin (Thermo Fisher Scientific, Inc.) at room temperature for 2 h and then were incubated with primary antibodies in 4°C for 12 h, followed by incubation with secondary antibodies at 25°C for 1 h and visualization with enhanced chemiluminescence reagent (Beyotime Biotechnology). ImageJ software (v1.52; National Institutes of Health) was used to quantify proteins. Primary antibodies included the following: Rabbit anti-FLNa antibody (1:1,000; cat. no. MAB1680; EMD Millipore), rabbit anti-EGFR antibody (1:1,000; cat. no. 18986-1-AP; ProteinTech Group, Inc.), rabbit anti-phosphorylated (p-)EGFR antibody (Tyr1068; 1:2,000; cat. no. 3777; Cell Signaling Technology, Inc.), rabbit anti-Akt antibody (1:1,000; cat. no. 10176-2-AP; ProteinTech Group, Inc.), rabbit anti-p-Akt antibody (Ser473; 1:1,000; cat. no. 66444-1-1g; ProteinTech Group, Inc.), rabbit anti-ERK1/2 antibody (1:4,000; cat. no. 16443-1-AP; ProteinTech Group, Inc.), rabbit anti-p-ERK1/2 antibody (Thr202/Tyr204; 1:3,000; cat. no. 4370; Cell Signaling Technology) and mouse anti-β-actin antibody (1:5,000; cat. no. 60008-1-1g; ProteinTech Group, Inc.). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:5,000; cat. no. 7076) and anti-rabbit IgG (1:5,000; cat. no. 7074) were used.

Xenograft experiments in vivo. A total of 20 BALB/c male nude mice (age, 6-8 weeks; weight, 15.3-18.2 g) were purchased from the Experimental Animal Center of Hebei Medical University. Mice were housed at 20-26°C and 40-60% humidity under a 12:12 h light/dark cycle, with access to food and water ad libitum. The present study was approved by the Laboratory Animal Ethical Committee of the Fourth Hospital of Hebei Medical University. Xenograft experiments were performed as previously described (33). Cells (2x10^6) suspended in Hank's balanced salt solution (HyClone; GE Healthcare Life Sciences) were injected in the right thigh. After 2 weeks, this experiment...
was ended. The isolated solid tumors were measured for size, weight and volume.

Patients and specimens. A total of 82 patients with CRC (51.1±15.9 years old, range, 42-74 years), who were admitted to the general surgery department at the 980th Hospital of the PLA Joint Logistics Support Force between November 2015 and May 2016, were included in the present study. All subjects provided written informed consent. Colorectal cancer tissues were obtained from the 980th Hospital of the PLA Joint Logistics Support Force (Bethune International Peace Hospital) with the approval of the Hospital Ethics Committee and were used according to ethical procedures.

Immunohistochemical (IHC) staining. Immunohistochemical staining was conducted to determine the protein expression of FLNa and Ki-67 by using a diaminobenzidine kit (OriGene Technologies, Inc.) according to the manufacturer's instructions. Tissues were fixed with 4% paraformaldehyde at room temperature for 48 h, embedded in paraffin and sectioned (5 µm). Briefly, after microwave-antigen retrieval, endogenous peroxidase activity was inhibited by incubation with 3% H₂O₂. Sections were blocked with 5% goat serum (Absin Bioscience, Inc.) at room temperature for 40 min, then incubated overnight at 4°C with a polyclonal rabbit anti-Ki-67 antibody (1:100; cat. no. 27309-1-AP; ProteinTech Group, Inc.) or anti-FLNa antibody (1:100; cat. no. 67133-1-Ig; ProteinTech Group, Inc.). After washing with PBS, the sections were incubated with a secondary antibody at 37°C for 30 min [HRP-conjugated goat anti-mouse IgG (1:1,000; cat. no. SA00001-1) and goat anti-rabbit IgG (1:1,000; cat. no. SA00001-2), both ProteinTech Group, Inc.]. Sections were counterstained with hematoxylin and eosin at room temperature for 4 min to visualize nuclei. Five different fields were randomly selected per sample. Images were acquired using a Leica microscope (magnification x100; Leica DM6000B; Leica Microsystems GmbH) and were digitized with LAS version 4.4 (Leica Microsystems GmbH). The thresholds for dichotomizing FLNa IHC were set to the medians for each measurement.

Statistical analysis. All data are reported as mean ± standard deviation. All statistics and graphs were obtained using GraphPad Prism 5 (GraphPad Software, Inc.); SPSS 19.0 software (IBM Corp.) was used for Kaplan-Meier plots and ANOVA. Cell viability, cell number, band density and gene expression were analyzed by ANOVA followed by Bonferroni post hoc test when making comparisons in datasets containing multiple groups. Spearman’s test was used for the correlation analysis of FLNa and Ki67. The maximal log-rank method...
Figure 2. FLNa knockdown promotes EGF-induced EGFR/Akt/ERK phosphorylation in SW480 cells. SW480 cells were transfected with shFLNa or control shRNA for 24 h. Then cells were treated with or without EGF (20 nM) for 0, 5, 10 and 30 min. (A) Total proteins were extracted and subjected to western blotting with antibodies against FLNa, EGFR, p-EGFR, p-Akt, Akt, p-ERK and ERK; β-actin is shown here as a loading control. (B) Band intensities for the ratio of FLNa/β-actin were measured. (C) Band intensities for the ratio of p-EGFR/EGFR, (D) p-Akt/Akt and (E) p-ERK/ERK were measured and normalized to β-actin. **P<0.01 vs. SW480/ctrl. FLNa, Filamin A; EGF, epidermal growth factor; sh, short hairpin; EGFR, epidermal growth factor receptor; p-, phosphorylated.

Figure 3. FLNa knockdown promotes tumor growth in vivo. (A) Representative image of xenograft tumors isolated from nude mice. Scale bar, 5 mm. (B) Tumor-growth curves. (C) Tumor weights at 21 days. (D) Western blot analysis of FLNa protein expression levels in xenograft tumors derived from SW480/KD and SW480/ctrl cells. Data are presented as mean ± standard deviation. *P<0.05 or **P<0.01 vs. SW480/ctrl (n=6 for each group).
was used for optimal cut-point determination and adjusted P-values. Cut-points within the central 80% of ordered FLna protein expression were considered. P<0.05 was considered to indicate a statistically significant difference.

Results

FLNa knockdown accelerates EGF-induced cell proliferation and migration in SW480 cells. To elucidate the role of FLNa in colon carcinoma cells, EGF-treated SW480 cells were transfected with shFLNa to knockdown FLNa expression. The efficacy of the knockdown was confirmed via western blotting. FLNa expression was significantly downregulated in the SW480/Kd group (0.48±0.01) compared with that of the SW480/ctrl group (1.13±0.03; P<0.01; Fig. 1 a). A cell viability assay demonstrated that FLNa knockdown promoted EGF-induced cell proliferation in a dose-dependent manner in the SW480/Kd group (P<0.01; Fig. 1B). Cell scratch migration assays demonstrated that FLNa knockdown significantly accelerated cell migration rates, and this effect was independent of EGF treatment (Fig. 1C and D). A Transwell assay also indicated that the number of migrated cells in the SW480/KD group was greater compared with the SW480/Control group (76±3 vs. 21±5; P<0.05) and the migration ability was even further increased in the SW480/KD group compared with those in the EGF-treatment group (Fig. 1E and F).

Effects of FLNa on EGFR phosphorylation in CRC cells. Because the activation of EGFR, Akt and ERK signaling is required for cell proliferation and migration (34), the effect of shRNA-mediated FLNa knockdown on EGF-stimulated SW480 cells was evaluated via western blotting. The protein expression levels of EGFR, Akt and ERK, and their activated phosphorylated forms, were detected in SW480/KD and SW480/Control cells treated with EGF for 0, 5, 10 and 30 min. FLNa expression in the SW480/KD group was significantly decreased at every tested time point compared with the control (P<0.01), indicating that FLNa knockdown activated the EGFR, Akt and ERK signaling pathways. These data suggested that FLNa regulated cell proliferation and migration via EGFR, Akt and ERK signaling in colon carcinoma cells.

FLNa silencing promotes SW480 cell growth in vivo. To further confirm the oncogenic efficiency of FLNa knockdown, SW480/KD or SW480/Control were subcutaneously inoculated into the left flank (SW480/KD) and right flank (SW480/Control in nude mice, respectively; n=8). After 14 days, the tumors were larger in the SW480/KD group compared with the SW480/Control group (0.613±0.114 vs. 0.378±0.068 cm³, P<0.05; Fig. 3 A and B). In addition, the tumor weight at the end of the experiment was increased in the SW480/KD group (0.512±0.031 g) compared with the SW480/Control group (0.371±0.044 g; P<0.05; Fig. 3C). Western blotting confirmed that the FLNa levels were significantly decreased in the

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Table I. Relationship between FLNa and clinical-pathological features.

| Characteristic              | N (%) | FLNa expression (%) | P-value |
|-----------------------------|-------|---------------------|---------|
|                            |       | Low (n=40)          | High (n=42) |
| Age ≤60                     | 38 (46.3) | 20 (50.0)                   | 18 (42.8) | 0.3928 |
| >60                         | 44 (53.7) | 20 (50.0)                   | 24 (57.1) | 0.9913 |
| Sex Female                  | 43 (52.4) | 21 (52.5)                   | 22 (52.4) | 0.6414 |
|                             | 39 (47.6) | 19 (47.5)                   | 20 (47.6) | 0.9827 |
| Diameter of tumor, cm ≤4   | 42 (51.2) | 20 (50.0)                   | 22 (52.4) | 0.0008 |
| >4                          | 40 (48.8) | 20 (50.0)                   | 20 (47.6) | 0.0007 |
| Degree of differentiation   |       |                      |         |
| Low                         | 37 (45.1) | 18 (45.0)                   | 19 (45.2) | 0.0008 |
| Moderate or high            | 45 (54.9) | 22 (55.0)                   | 23 (54.8) | 0.0008 |
| TNM stage                   |       |                      |         |
| I-II                        | 36 (43.9) | 10 (25.0)                   | 26 (61.9) | 0.0008 |
| III-IV                      | 46 (56.1) | 30 (75.0)                   | 16 (38.1) | 0.0008 |
| Lymph node metastasis       |       |                      |         |
| Yes                         | 48 (58.5) | 31 (77.5)                   | 17 (40.5) | 0.0008 |
| No                          | 34 (41.5) | 9 (22.5)                    | 25 (59.5) | 0.0008 |

FLNa, Filamin A; TNM, Tumor, Node, Metastasis.
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Table II. Relationship between the expression of FLNa and Ki-67.

| FLNa expression | Ki-67 expression (%) | rs  | P-value |
|----------------|----------------------|-----|---------|
| Negative (n=40)| 12 (31.6)            | -0.32| 0.004   |
| Positive (n=42)| 26 (68.4)            |     |         |

FLNa, Filamin A.

Figure 4. Expression of FLNa in CRC tissues and patient survival. (A) Representative photographs of hematoxylin and eosin and (B) immunohistochemical staining for FLNa and Ki-67 in CRC tissues and adjacent normal tissues (magnification, x100). (C) Bar graph showing significantly higher expression of Ki-67 and lower expression of FLNa in CRC tissues compared with adjacent normal tissues. Data are presented as mean ± standard deviation. *P<0.05 vs. normal. (D) Patients were divided into two groups, low or high FLNa expression, based on the minimum observed P-value. The overall survival of CRC patients with low and high FLNa expression was evaluated using Kaplan-Meier survival analysis (log-rank P=0.056). FLNa, Filamin A; CRC, colorectal cancer.

SW480/KD tumor tissues compared with those in the control (Fig. 3D). These results further indicated that FLNa knockdown accelerated CRC tumor growth.

Expression of FLNa in CRC tissues from patients and correlation with Ki-67 expression. The characteristics of the 82 CRC patients (median, 51.1±15.7 years; range, 42-74 years old) enrolled in the present study are listed in Table I. The expression levels of FLNa exhibited a significant association with TNM stage and lymph node metastasis in CRC, but not with gender, age, diameter of tumor, or degree of differentiation (Table I). Representative photographs of hematoxylin and eosin and immunohistochemical staining for FLNa and Ki-67 in adjacent normal tissues and CRC tissues (Fig. 4A and B) revealed significantly higher expression of Ki-67 and lower expression of FLNa in CRC tissues compared with adjacent normal tissues (P<0.05; Fig. 4C). FLNa expression had a significant negative correlation with Ki-67 expression (Table II), which may explain how FLNa knockdown promoted tumor growth. By contrast, low expression of FLNa did not influence the overall survival time for the patients with CRC, as evidenced by Kaplan-Meier survival analysis (Fig. 4D).
Discussion

As a large cytoplasmic protein, FLNa mainly crosslinks actin filaments, membrane receptors and signaling intermediates (35). The specific role of FLNa in cancer metastasis has remained elusive. Through MTS, wound healing and Transwell assays in the present study, it was identified that FLNa knockdown increased the proliferative and migratory abilities of CRC cells in vitro. In addition, FLNa knockdown was demonstrated to promote tumor growth in vivo. These findings are in accord with an earlier report on breast cancer cells that overexpressed ErbB2, in which knockdown of FLNa promoted division and metastasis of tumor cells (36).

CRC cells invade and metastasize to distant sites and these phenomena are accompanied by aberrant activation of cell signaling (37). A number of studies have reported filamin structures and filamin-binding proteins that are involved in cell signaling and other important cellular functions. For example, FLNa is involved in the organization of the actin network (10,38), acts as a ‘molecular switch’ to convert mechanical stimuli to chemical signals (39). These multiple interactions suggest that FLNa is a key component of a variable signaling-scaffold complex (10). In addition, EGFR is an actin-binding protein and has become an indispensable molecular target for cancer therapy. EGFR is activated by certain ligands (such as EGF) and provokes various cellular signaling events resulting in increased cell proliferation, invasion and metastasis (29). EGFR carries out these functions through activating multiple signaling cascades, including RAS/RAF/MEK/ERK and PI3K/Akt pathways (40). The present study demonstrated that the phosphorylation levels of EGFR, ERK and Akt were significantly increased in the FLNa-knockdown group compared with the control, whereas no difference was observed in the total EGFR, ERK and Akt expression levels between these two groups. The present results indicated that lower expression of FLNa promoted the proliferative and migratory ability of CRC cells by activating EGFR and its downstream signaling proteins. Under certain circumstances, this aberrantly activated pathway may lead to abnormal cell growth and invasion, which could subsequently result in drug resistance or metastasis to distant sites (41). An important limitation of the present study was that only one cell line was used; therefore, future studies are required to confirm the findings of the present study by using additional cell lines.

Accumulating evidence suggests that FLNa participates in the development of multiple tumors. Xu et al (42) demonstrated that FLNa regulates focal-adhesion disassembly and inhibits breast cancer cell migration and invasion. A previous study showed that there is a close relationship between FLNa and VEGF in patients with lung cancer (43). However, a separate study emphasized that FLNa interacting with other proteins inhibits CRC progression (44), indicating that FLNa may represent a novel cancer-suppressor gene for treating colorectal adenocarcinoma. Thus, the current literature suggests a dual role for FLNa and complex underlying mechanisms in various types of cancer. The present study found that FLNa knockdown correlated with TNM stage and lymph node metastasis in patients with CRC, which is in accordance with a previous study on gastric cancer (45). A possible mechanism for the down-regulation of the FLNa gene in CRC cells may be mutagenesis.

Notably, FLNa expression was demonstrated to be negatively correlated with the expression of Ki-67, which is a reliable index of cancer progression. The present data suggested that FLNa may have protective roles as a negative regulator in CRC SW480 cells by promoting proliferation via the activation of many signaling pathways. Hence, FLNa may represent a novel prognostic marker and therapeutic target for treating CRC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

KW performed the experiments and data analysis. KW and TZ participated in the design and coordination of experimental work. KW and RJZ were involved in data acquisition. KW and TZ drafted the manuscript.

Ethics approval and consent to participate

The present study was approved by the Laboratory Animal Ethics Committee of the Fourth Hospital of Hebei Medical University (Shijiazhuang, China). Experimental procedures were implemented in accordance with the guidelines and regulations of the Hospital Ethics Committee and were performed according to ethical procedures of the 980th Hospital of the PLA Joint Logistics Support Force (Bethune International Peace Hospital).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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