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Increased expression of formin-like 3 contributes to metastasis and poor prognosis in colorectal carcinoma

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ABSTRACT

Formin-like 3 (*FMNL3*), a member of diaphanous-related formins subfamily, plays an important role in cytoskeleton reorganization, cell adhesion and cancer cell invasion in vitro. This study aimed to explore the expression of *FMNL3* in colorectal carcinoma (CRC) cell-lines and tissues, and further evaluate its prognostic value and correlation with the clinicopathological parameters, and also investigate the effects of *FMNL3* gene silencing on the growth and metastasis of CRC in vivo. Immunohistochemical analysis showed that *FMNL3* protein was distributed in a punctuate aggregation pattern and located mainly in the cytoplasm of glandular cavity side, close to the nucleus of CRC cells. The positive rate of *FMNL3* expression was 87.5% (84/96) in CRC, which was significantly higher than that in adjacent normal mucosa (30%, 9/30). Moreover, *FMNL3* protein expressed far more in primary CRC with metastasis and corresponding lymph nodes metastatic CRC than in primary CRC without metastasis. Increased expression of *FMNL3* was closely correlated with tumor size, differentiation, serosal invasion, and both lymph node metastasis and distant metastasis. However, it was not correlated with patients' age and gender. According to Kaplan–Meier survival analyses, patients with *FMNL3* high expression level had lower overall survival rate than that with *FMNL3* low expression level. Univariate and multivariate analyses revealed that high *FMNL3* expression was a significant and independent prognostic predictor of patients with CRC. In addition, *FMNL3* mRNA and protein levels were substantially up-regulated in CRC-metastasis-derived cell lines, as compared to those in primary-CRC-derived ones. *FMNL3* gene silencing suppressed the growth and metastasis of CRC in vivo. In conclusion, *FMNL3* plays an important role in the progression and metastasis of CRC and may be a novel potential prognostic predictor and therapeutic target for patients with CRC.

1. Introduction

Colorectal carcinoma (CRC) is one of the most common malignancies in humans with over one-million new cases diagnosed worldwide every year (Harrison and Benziger, 2011) and over 50% of patients will develop hepatic metastasis during the course of the disease (Manfredi et al., 2006). The 5-year survival rate in early CRC patients is approximately 90%; however, the rate decreases to less than 10% in patients with distant metastases (Shen et al., 2007). Although various multidisciplinary therapies including chemotherapy, surgery, and regional therapy either alone or in combination, have improved the survival rate in patients with metastatic CRC (Guye et al., 2013), the prognosis in patients still remains poor (Coppede et al., 2014). Therefore, it is essential to identify novel effective therapeutic targets and prognostic biomarkers to prevent CRC-related deaths.

Tumor metastasis is a highly complicated, multi-step process, which involves cell migration through tissues, dynamic interaction with extracellular matrix and rearrangement of cell–cell contacts (Sahai, 2007). The steps involving metastasis depend on the cytoskeleton reorganization. Formins are ubiquitous and highly conserved multi-domain proteins that govern cell shape, cytokinesis, adhesion and motility by remodeling actin cytoskeleton through their formin homology 2 (FH2) domains (Eisenmann et al., 2007; Faix and Grosse, 2006; Wodarz and Nathke, 2007). Diaphanous-related formins (DRFs), a conserved subfamily of formins, increase the signaling complexity of formins because of their ability to act as effectors of Rho guanine triphosphatases

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(GTPases) (Goode and Eck, 2007). Recently, DRFs were reported to be deregulated in tumor cell transformation and metastasis (Hager et al., 2012; Zhu et al., 2008).

Formin-like 3 (*FMNL3*), a member of the DRFs subfamily, is mapped to human chromosome 12q13 (Katoh and Katoh, 2003). Although several groups have conducted in-depth studies on the effects of *FMNL3* in regulating cytoskeleton reorganization, cell–cell adhesion and cancer cell invasion in vitro (Gauvin et al., 2015; Harris et al., 2010; Hetheridge et al., 2012; Lynch et al., 2013; Moriya et al., 2012; Thurston et al., 2012; Vega et al., 2011). However, the role of *FMNL3* in the clinical progression and metastasis of malignant tumor remains unknown. In this study, we have investigated the expression of *FMNL3* in CRC cell-lines and tissues, and evaluated its prognostic value and correlation with clinicopathologic parameters of patients with CRC. In addition, we have explored the effects of *FMNL3* gene silencing on the growth and metastasis of CRC cells in vivo.

2. Materials and methods

2.1. Patients and tissue samples

A total of 96 patients with primary CRC, who had undergone routine surgery but were not given radiotherapy or chemotherapy before surgery at Jiangxi Provincial People's Hospital (China) from 2006 to 2008, were enrolled for the study. The participants were followed-up for 5 years. The relevant clinical data were described in Table 1. The CRC tissue samples were fixed in 10% formalin and were embedded in paraffin. The tissues were then sectioned serially at 4 μm and were stained by immunohistochemistry (IHC) with anti-*FMNL3* antibody. The sections were then assigned to three pathologists to read independently in a double-blinded manner, according to the World Health Organization (WHO) criteria. The research protocol was performed in accordance with the ethical standards and was approved by the Ethics Committee of Jiangxi Provincial People's Hospital in Nanchang, and informed consent was obtained from all individual participants included in this study.

Table 1
The relationship between FMNL3 expression and clinicopathological parameters in patients with CRC.

Features	Total number	High expression	Low expression	*P* values	*Z* values
All cases	96	61	35		
Age (years)					
<60	50	32	18	0.923[a]	−0.097
≧60	46	29	17		
Gender					
Male	58	33	25	0.096[a]	−1.662
Female	38	28	10		
Tumor size (cm)					
<3	29	14	15	0.042[a*]	−2.034
≧3	67	47	20		
Differentiation					
Well	31	18	13	0.028 [a*]	−2.201
Moderate	42	22	20		
Poor	23	21	2		
Serosal invasion					
Yes	61	44	17	0.022 [a*]	−2.296
No	35	17	18		
Lymph node metastasis					
Yes	37	29	8	0.017[a*]	−2.379
No	59	32	27		
Distant metastasis					
Yes	17	16	1	**0.004**[a*]	−2.872
No	79	45	34		

* Statistically significant difference ($P < 0.05$).
[a] Mann–Whitney *U* test.

2.2. Cell-lines

All 6 CRC cell lines (LoVo, SW620, SW480, LS174T, HT29 and HCT116) were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). The two cell lines SW480 and HCT-116, were maintained in RPMI 1640 (Hyclone) supplemented with 10% FBS (Gibco), whereas SW620 and LS174T were grown in DMEM (Hyclone) supplemented with 10% FBS. The remaining cell lines LoVo and HT29 were maintained in F12K and McCOY'S 5A respectively (Sigma), supplemented with 10% FBS. All the cell lines were incubated in a 5% CO_2-humidified atmosphere at 37 °C.

2.3. Animals

Three-to-four-week old immunodeficient BALB/c nude mice were purchased from Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). The mice were raised in a specific-pathogen-free (SPF) environment at laboratory animal rooms of Institute of Translational Medicine in Nanchang University (China). The protocols for animal studies were performed in accordance with the ethical standards and were approved by the Institutional Animal Care and Use Committee in Nanchang University.

2.4. Immunohistochemical staining and evaluation

Ninety-six tissue samples were formaldehyde-fixed and were embedded in paraffin. The tissues were sectioned (4 μm) and then deparaffinized and rehydrated. Following this, the tissues were antigen retrieval in sodium citrate buffer (10 mM, pH: 6.0) in a pressure cooker for 5 min. Then they were blocked with 5% normal goat serum, and were incubated with 1:100 anti-*FMNL3* antibody (Abnova) for 1 h and incubated with HRP-conjugated goat secondary antibody (Maixin) for 15 min at room temperature. The visualization signals were developed using DAB and the samples were counterstained with hematoxylin. All the results of IHC staining for *FMNL3* were scored separately by three pathologists, blinded to the clinical information as previous study (Soumaoro et al., 2004). The staining intensity was recorded as 0 (negative), 1 (weak), 2 (medium), and 3 (strong). The extent of staining was scored as 0 (0%), 1 (1%–25%), 2 (26%–50%), 3 (51%–75%), and 4 (76%–100%), according to the percentage of stained area in relation to the entire carcinoma-involved area or the entire section for the normal samples. The intensity and extent scores were combined to produce the final staining score (0–7) for *FMNL3* expression. The staining of *FMNL3* was assessed as follows: (−): final staining score of <3; (+): final score of 3; (++): final score of 4; and (+++): final score of 5 or more. Tumors having a final staining score of 3 or higher were considered to be positive. Cutoff values for *FMNL3* were chosen based on the heterogeneity level using log-rank statistical analysis with respect to overall survival. An optimal cutoff value was identified as: tumors with a final staining score of − or + were classified as having low expression of *FMNL3*; and tumors with a final staining score of ++ or +++ were classified as having high *FMNL3* expression.

2.5. RNA extraction and real-time polymerase chain reaction

Total RNA was extracted using Trizol reagent (Life technology, USA), and cDNA was synthesized by oligo (dT)-primed reverse transcription from 1 μg of total RNA using an access reverse transcription (RT) system (TaKaRa). Reverse transcription (RT) was carried out for 20 min at 42 °C. For real-time polymerase chain reaction (PCR), we used LightCycler® 96 Real-time RT-PCR System (Roche) and SYBR® Premix Ex Taq (Takara) with the following thermal cycling profile: 95 °C for 30 s, followed by 40 cycles of amplification (95 °C for 5 s, 60 °C for 20 s), and melt curve analysis to validate the amplification of single product. Each reaction mixture consisted of 10 μL of 2 × SYBR® Premix ExTaq II, 1 μg of cDNA template, 0.8 μL PCR forward primer (10 μmol/L),