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Original Research Article

FXR1 knockdown inhibits the malignant behavior of colorectal cancer by suppressing epithelial-to-mesenchymal transition

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Abstract

Purpose: *To determine the role of Fragile X-related protein-1 (FXR1) in colon cancer progression and its relationship to patients' survival.*
Methods: *A total of 164 colorectal cancer (CRC) patients, admitted to the Affiliated Hospital of Jiangnan University, Wuxi, Jiangsu, China between 2006 and 2008, were included in this study. Immunohistochemistry was used to semi-quantitatively analyze the intensity and extent of immunological staining of diaminobenzidine-stained paraffin blocks of CRC samples. The study also retrieved COAD mRNA and patients' clinical data from TCGA and cultured human colon cancer cell lines (SW480, SW620, HCT8, HCT116, and Caco2) in RPMI 1640 medium to assess the propensity of CRC cells to proliferate, invade the tumorigenicity in BALB/c nude mice.*
Results: *The prognosis of CRC patients was inversely linked with the expression of FXR1. Additionally, FXR1 knockdown in CRC cells reduced cellular growth, colony development and tumorigenesis. After presenting BALB/c nude mice with tumors in FXR1 knockdown, the cells displayed higher E-cadherin levels ($p < 0.01$) as well as decreased TGF-1 ($p < 0.01$) and N-cadherin levels ($p < 0.001$).*
Conclusion: *Fragile X-related protein-1 is an oncogene in colon cancer and its knockdown inhibits HCT116 cells from behaving malignantly. Thus, FXR1 is a potential treatment option for CRC.*

Keywords: *Colorectal cancer, FXR1, EMT*

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INTRODUCTION

According to the WHO International Agency for Research on Cancer (IARC) most recent global statistics for 2020, colorectal cancer (CRC) has the second-highest mortality rate of all cancers and the third-most prevalent worldwide. By 2030, it is anticipated that there will be 2.2 million new cases and 1.1 million deaths due to CRC [1].

Over the last few decades, CRC incidence and death have gradually increased in China. Because of lack of symptoms during the early stages, 50 to 60 % of all CRC patients suffer

recurrences and metastases [1,2]. Furthermore, collecting a large number of mutations and/or epigenetic changes can drive tumor formation and development. The aberrant expression of RNA-binding proteins has profoundly impacted cellular physiology and could alter gene expression and promote carcinogenesis among multiple cancers, including CRC [3-5].

Fragile X-related protein-1 (FXR1) appears to be a crucial regulator of tumor development and is required for the formation of non-small cell lung cancer as well as head and neck squamous cell carcinoma [6,7]. Poor clinical outcomes have been documented for CRC patients with increased plasma FXR1 levels. However, the functional role of FXR1 in CRC remains elusive [8]. Therefore, this study seeks to assess the clinical significance of FXR1 in CRC by exploring its effect on the malignant behavior of CRC. Results from this study show that FXR1 promotes the progression of CRC and opens up new perspectives for further investigation of the mechanisms that act on the development of CRC.

METHODS

CRC tissue samples

Tissue samples were taken from 164 colorectal cancer patients who underwent surgical resection at the Affiliated Hospital of Jiangnan University, Wuxi, Jiangsu, China between 2006 and 2008. The research only included instances that were verified by histology. Patients who had received chemotherapy or radiation previously, as well as those with insufficient clinical data, were excluded. The pathological stages of all the patients were assessed according to the guidelines of American Joint Committee on Cancer (AJCC, 7th Ed) staging system, based on available clinical and pathological data regarding tumor size, nodal involvement, and metastases. Two experienced pathologists evaluated the histopathological features and selected up to three representative tumor areas from hematoxylin and eosin-stained sections. Patients' demographic characteristics such as age and gender, as well as pathological characteristics such as histological grade, vascular invasion, neural and lymphatic invasion, Lauren classification and serum carcinoembryonic antigen (CEA) levels, were recorded.

Ethical matters

The Medical Ethics Committee of the Affiliated Hospital of Jiangnan University in China approved this study (approval no. JN-A-07). All animal studies were carried out in compliance with the guidelines established by Jiangnan University's Animal Ethics Committee. Both the human and animal studies followed the international guidelines for human and animal studies, respectively.

Staining of immunohistochemistry

Paraffin blocks of CRC samples were split into 4 µm pieces. The sections were then deparaffinized with xylene, dehydrated with ethanol, rinsed with distilled water and permeabilized for 30 min in 0.3 % hydrogen peroxide. The slides were submerged in a 95 ^{o}C citrate buffer solution for 10 min for antigen retrieval. After cooling, the slides were immersed in hydrogen peroxide and blocking solution and were exposed to a primary antibody for overnight incubation at 4 ^{o}C. Diaminobenzidine (DAB, Solarbio, Beijing, China) was used to determine the target molecule's expression and cell nuclei was stained with hematoxylin. The slides were dehydrated using gradient alcohol and xylene after being washed in running buffer. Finally, a cover glass was placed over the neutral balsam on the glass slide. The slides were then stored in a cold, dry environment before being scanned with a Panoramic MIDI slide scanner.

Evaluation of immunohistochemistry

Sections were independently reviewed by two separate pathologists. The intensity and extent of immunological staining were assessed semi-quantitatively. The intensity of the staining was determined as follows: 0 represents zero staining, 1 represents weakly positive staining, 2 represents moderately positive staining and 3 represents highly positive staining. The proportion of positively stained tumor cells was graded: 5 % received a score of 0, 6 to 25 % received a score of 1, 26 to 50 % received a score of 2, and 51 % received a score of 3. If the intensity multiplied by the percentage score resulted in a total score of less than 3, the protein level was termed poor. Protein expression, on the other hand, was regarded as high if the score was 4 or greater.

Integrated-signature FXR1 analysis of TCGA

The COAD mRNA data and patient's clinical data (tumor tissue and/or surrounding normal tissue) were collected from TCGA (https://tcgadata.nci.nih.gov/docs/publications/tcga/; up to January 1, 2018) and BRB array tools (version 4.3.2; National Cancer Institute, Bethesda, MD, USA) were used for the expression analysis.

Cell culture

American Type Culture Collection (ATCC, Manassas, VA, USA) provided the Human colon cancer cell lines (SW480, SW620, HCT8, HCT116, and Caco2). Cells were grown in RPMI 1640 media (Gibco) supplemented with 10 % fetal bovine serum (FBS; Invitrogen) and 1 % penicillin (100 g/mL) in a 5 % CO_2 environment at 37 ℃. When the confluence reached > 90 %, the cells were passaged at an acceptable density.

Cell proliferation assay

The CCK-8 test (Yeasen, Shanghai, China) was used to evaluate the rate of cell proliferation based on manufacturer's recommendations. Absorbance was read using a Microplate reader (Bio-Rad, 450 nm wavelength).

Colony-forming assay

Cells were seeded in triplicate on a 24-well plate with a basal layer of 0.8 mL growth medium containing 0.6 % low melting temperature agarose (Promega, Madison, WI, USA). Until colonies (C) were formed, plates were cultured for 12 - 14 days. One positive colony was defined as one with a diameter greater than 75 mm or more than 50 cells. The formula used to determine clone creation efficiency (CE) is shown in Eq 1.

CE = (C/cI)100 …….. (1)

Where cI represents cells injected

Real-time quantitative PCR

The RNA was isolated using a Cell/Tissue total RNA isolation kit (Yeasen, Shanghai, China). A 1 µg RNA was reverse transcribed into cDNA as described by the manufacturer. Quantitative PCR (qPCR) was performed using SYBR Green 2× PCR mix with 40 cycles by LightCycler 480 (Roche, San Francisco, USA). Relative mRNA levels were standardized to β-ACTIN. The primers used are shown in Table 1.

Table 1: The primer sequence

Gene	Sequence
FXR1 forward	5′- GGCTAAAGTCGGATGATG -3′
FXR1 reverse	5′-ATGAAAAGCTGCTGCAAG -3′
β-ACTIN forward	5′-TGACGTGGACATCCGCAAAG-3′
β-ACTIN reverse	5′- CTGGAAGGTGGACAGCGAGG-3′

Western blotting and antibodies

Approximately 10^6 cells were harvested and lysed with protein extraction buffer (RIPA buffer, Beyotime, Shanghai, China) and PMSF for western blot analysis, as described previously [9]. A bicinchoninic acid (BCA) protein determination kit (Beyotime, Shanghai, China) was used to determine protein concentration. The SDS-PAGE was used to separate 30 g of protein, which was then deposited onto 0.45 µm PVDF membranes (Millipore, MA, USA). Membranes were incubated for 1 h with 5 % Difco skim milk (BD, NJ, USA), followed by primary antibodies at 4 ℃ overnight, secondary antibodies (Absin, Shanghai, China) at room temperature for 1 h and finally determined using enhanced chemiluminescence (ECL) detection kit (NCM biotech, Suzhou, China). The antibodies used for western blot were FXR1, TGF-β, E-Cadherin, and N-Cadherin (Abcam, Waltham, MA, USA) and GAPDH (BD Biosciences, Palo Alto, CA, USA).

Construction of FXR1 shRNA Lentiviral vector

The FXR1 shRNA lentiviral plasmids were synthesized and purified by Hanheng Biotechnology Co. Ltd (Shanghai, China). ShFXR1 plasmids were co-transfected using pSPAX2 and pMD2G plasmids into 293T cells. Six hours after transfection, the medium was changed to a new one. After harvesting cells at 48 and 72 h later, the supernatant of transfected cells was centrifuged at 3000 rpm for 10 min before being used to transfect HCT116 cells.

The shRNA sequence is as follows:

Top strand:
GatccGCGCCAGGTTCCATTTAATGAATTCAAG
AGATTCATTAAATGGAACCTGGCGTTTTTTc

Bottom strand:
AattgAAAAAACGCCAGGTTCCATTTAATGAAT
CTCTTGAATTCATTAAATGGAACCTGGCGCg

Animal studies

BALB/c nude male mice (6 – 7 weeks old) were obtained from Beijing Vital River Laboratory Animal Technology Co. (Beijing, China). Intradermal injections of FXR1-knockdown HCT116 cells (5 x 10^5 cells/0.1 mL medium) were administered to each mouse's left and right backsides. Four weeks after the injections, the mice were euthanized after being checked daily for tumor progression.

Table 2: Correlation between FXR1 expression and clinic pathological characteristics of colorectal cancer

Characteristic	Cases	FXR1 expression		*P*-value
		Low (n=83)	High (n=81)	
Gender				0.876
Male	82	41	41	
Female	82	42	40	
Age (mean=62)				***0.695***
<62	81	42	39	
≥62	83	41	42	
Clinical stage				***0.162***
I-III	88	49	39	
III-IV	76	34	42	
T				***0.436***
T1-2	45	25	20	
T3-4	119	58	61	
N				***0.766***
N0	91	47	44	
N1-2	73	36	37	
M				***0.049****
MO	144	77	67	
M1	20	6	14	
Differentiation				***0.154***
Moderate & Well	128	61	67	
Poor	36	22	14	

*$p < 0.05$

Statistical analysis

The most appropriate cut-off value for FXR1 histopathological scores was determined by ROC curve analysis. Pearson's chi-square or Fisher's exact test was used to assess the association between FXR1 expression and clinicopathological parameters.

Kaplan-Meier method was used to estimate survival curves and data was analyzed using the log-rank test. To find possible risks variously linked to the results, univariate analyses were conducted. Significant clinicopathological variables were included in subsequent multivariate analyses. A logistic regression model was applied to measure the area under the ROC curve (AUC) and to evaluate the predictive effect of multiple variables on the prognostic outcomes of CRC patients. All the tests were 2-sided and statistical significance was defined as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$, as appropriate.

RESULTS

FXR1 protein expression is high in human CRC tissues

A total of 164 CRC tissue samples were collected from 82 male and 82 female CRC patients. Clinicopathological and demographic characteristics are summarized in Table 2. Patients' average age at the time of surgery was 62. Patients were at different stages of tumor progression viz – T1 (2 patients), T2 (43 patients), T3 (78) and T4 (41 patients). Positive lymph node metastases were observed in 73 patients, while distant metastases were recorded in 20 patients. Based on TNM staging system, 45 patients were at stages I/II, while 119 were at stages III/IV.

FXR1 protein was determined by immunohistochemistry so as to compare its expression in colon cancer versus adjacent tissues. Results revealed that FXR1 protein levels in colon cancer tissues were significantly higher than in adjacent tissues ($p < 0.05$). The FXR1 protein was predominantly found in tumor cell nuclei, cell membranes, and interstitial fluid. Eighty percent of 164 cases of colon cancer tissues were positive for FXR1 expression compared to only 37 % of normal adjacent tissues (164 cases in total, $p = 0.007$) (Figure 1 A). The RT-PCR was used to assess FXR1 mRNA levels in 27 paired samples of CRC and nearby tissues. The results revealed that FXR1 mRNA expression was considerably higher in cancer samples than in surrounding tissues (Figure 1 B and C).

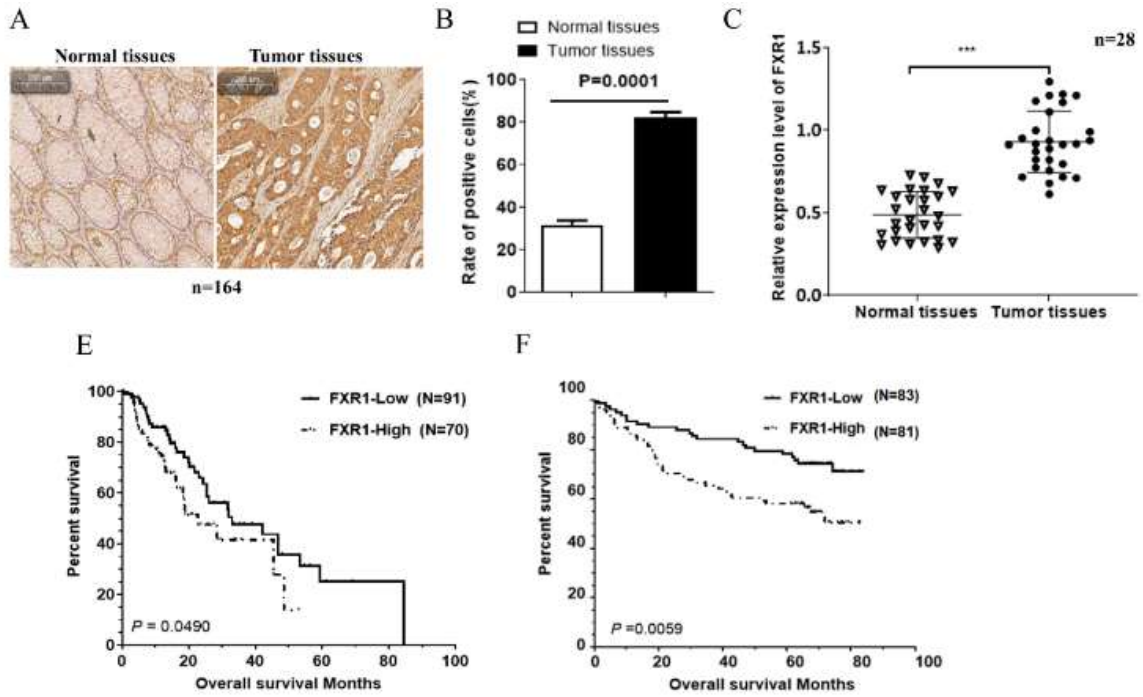

Figure 1: FXR1 is substantially expressed in CRC tissues (A, B) Immunohistochemical analysis of FXR1 in CRC and nearby tissues (C) RT-PCR analysis of FXR1 level in CRC and nearby tissues (D) Data gleaned from TCGA (E) Information from 164 CRC patients who underwent surgical resections

Expression of FXR1 is inversely linked with the prognosis of CRC patients

FXR1 protein was strongly linked with M-stage colon cancer (p = 0.049). Gender (p = 0.876), age (p = 0.695), clinical stage (p = 0.162), T stage (p = 0.436), N status (p = 0.766) and differentiation (p = 0.154) were all not shown to be significantly associated.

Kaplan-Meier analysis of the TCGA dataset indicated that patients with higher FXR1 expression levels demonstrated poorer outcomes (p = 0.0490) than those with lower FXR1 (Figure 1 D). Survival curves in low and high FXR1 expression groups are represented in Figure 1 A. With 5-year survival rates of 62 and 83 % respectively, in the FXR1-high group compared to the FXR1-low group (p = 0.0059, Figure 1 E), overall survival was considerably lower in the FXR1-high group. According to the findings, CRC patients' poor prognoses are significantly predicted by high FXR1 expression.

In addition, the univariate analysis identified five statistically significant prognostic factors: FXR1 protein level (p = 0.007), differentiation (p = 0.015), T stage (p = 0.024), N status (p = 0.001) and M stage (p < 0.001). The other clinicopathological characteristics, such as location, age and gender were not statistically significant (Table 2). Moreover, multivariate analysis established that high FXR1 protein levels (p = 0.014), tumor differentiation (p = 0.002), N status (p = 0.003) and M stage (p = 0.001) were independent predictors of poor overall survival among CRC patients (Table 3).

FXR1 is highly expressed in human CRC cell lines

The results showed that FXR1 was highly expressed among all the selected cell lines, with HCT116 cells depicting the highest values (data not shown). Therefore, HCT116 cells were chosen for RNA interference experiments to investigate the influence of FXR1 on biological characteristics of intestinal cancer cells.

Down-regulation of FXR1 reduced proliferation, invasion and clone formation

Results show that shFXR1-HCT116 cells' capacity for proliferation was inhibited (Figure 2 A). However, no statistically significant alterations were seen between HCT116 (HCT116/WT) and shNC-HCT116 cells. Similarly, Transwell invasion assay and colony-forming results revealed that these capabilities were significantly suppressed in shFXR1-HCT116 (Figures 2 B - D). These results show that when FXR1 was down-regulated, HCT116 cells significantly reduced their ability to proliferate, invade, and form colonies.

Table 3: Univariate and multivariate analysis for overall survival (Cox proportional hazards regression model)

Rink factors	Univariate			Multivariate		
	HR	*P*-value	95% CI	HR	*P*-value	95% CI
FXR1(low/high)	2.072	0.007*	1.220-3.518	2.036	0.014*	1.153-3.594
Age (<62/≥62)	1.072	0.789	0.643-1.787	1.315	0.318	0.768-2.251
Gender(male/female)	0.894	0.668	0.536-1.491	0.719	0.233	0.419-1.236
T(T1-2/T3-4)	2.192	0.024*	1.110-4.329	1.924	0.072	0.911-3.922
N (N0/N1-2)	2.743	<0.001*	1.614-4.661	2.384	0.003*	1.331-4.268
M (M0, M1)	6.640	<0.001*	3.690-11.948	5.718	<0.001*	2.987-10.945
Location (left or right)	1.452	0.159	0.862-2.479	1.289	0.38	0.731-2.273
Differentiation (moderate and well/poor)	1.978	0.015*	1.145-3.419	2.570	0.002*	1.396-4.732

*HR: Hazard ratio, *p < 0.05, left: left colon, right: right colon*

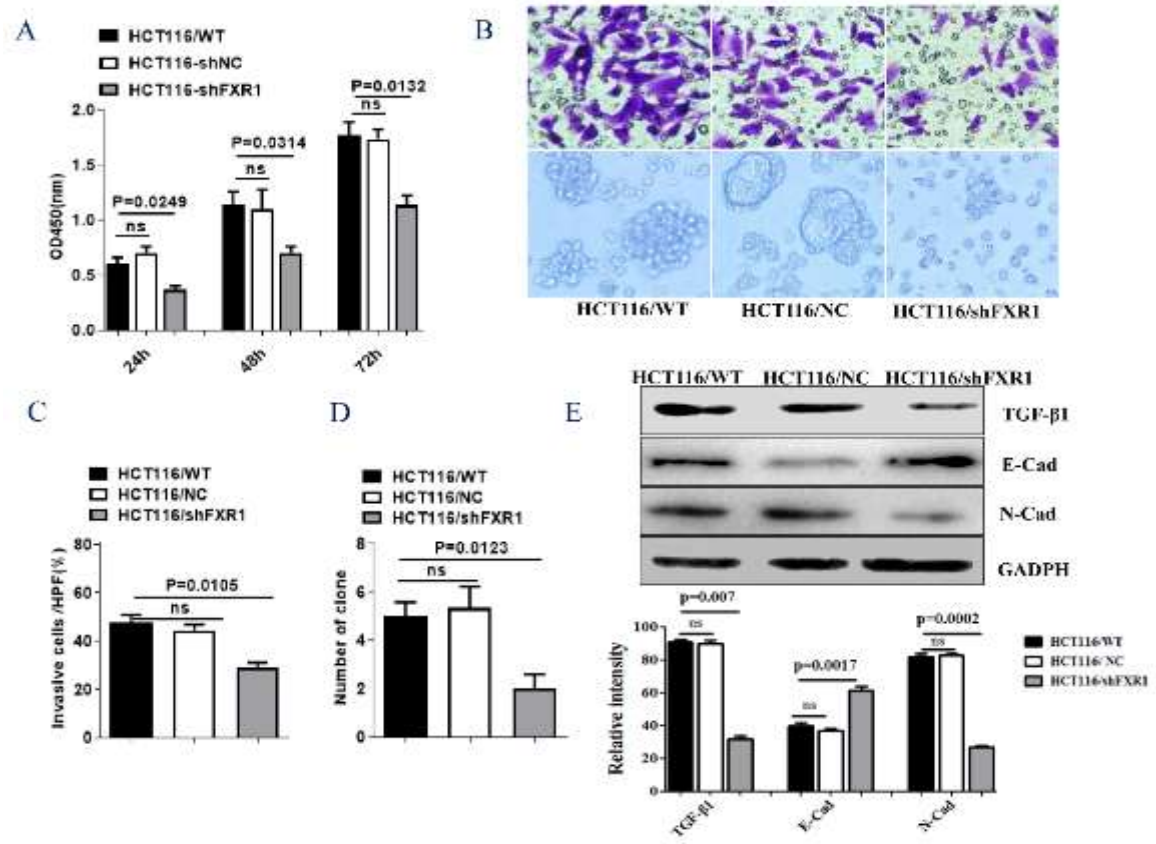

Figure 2: Down-regulation of FXR1 reduced the growth of CRC *in vitro*. (A) Cell proliferation in differently treated HCT116 cells. (B) Soft-agar colony formation in HCT116 cells under various circumstances was depicted in the photographs. (C) A statistical examination of colony-forming rates. (D) Transwell invasion assay of HCT116 cells under various conditions. (E) Western blot analysis of FXR1, N-cadherin, E-cadherin, and TGF-β in differentially treated HCT116 cells. Semi-quantitative analysis of these levels revealed statistically significant changes for N-cadherin (p = 0.007), E-cadherin (p = 0.0017) and TGF-β (p = 0.0002)

Down-regulation of FXR1 reversed the Epithelial-to-Mesenchymal Transition (EMT) of HCT116 cells

The complexity and significance of the EMT have been significantly appreciated across various cancer types in recent years. EMT, in which N-cadherin is a mesenchymal indicator and E-cadherin is an epithelial indicator, is influenced and promoted by the TGF-β pathway. This study further investigated these markers and pathways in FXR1 knockdown HCT116 cells. In parallel with colony-forming and invasion assays, the mesenchymal and epithelial indicator levels were evaluated in shFXR1-HCT116 cells using Western blot. The results revealed that the expression of TGF-β and N-cadherin was reduced in the knockdown cells, while E-cadherin expression was significantly up-regulated (Figure 2 E). These results revealed that the down-regulation of FXR1 reversed the EMT process.

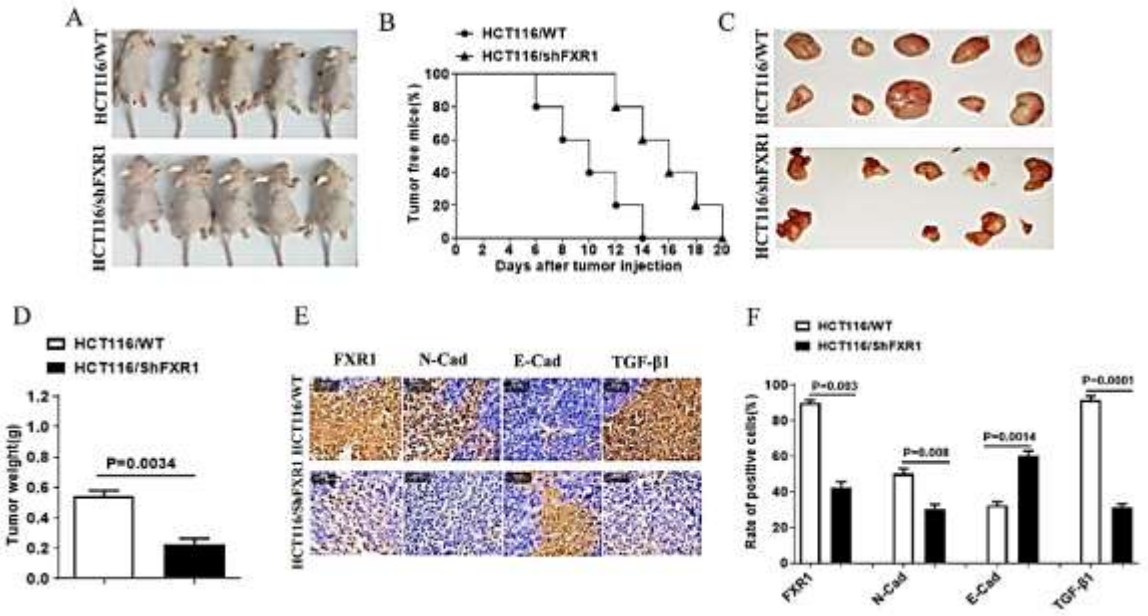

Figure 3: Knockdown of FXR1 attenuates the tumorigenesis of CRC cells. (A) Images of mice bearing subcutaneous tumors from the differentially treated HCT116 cells. (B) Tumor-free mice at 28 days. (C) Sizes of HCT116 and shFXR1-HCT116 tumors. (D) Weight of the HCT116 and shFXR1-HCT116 tumors. (E) Immunohistochemical analysis depicting representative images of HTC116 with or without FXR1 knockdown cells expressing FXR1, N-cadherin, E-cadherin, and TGF-β (×400). Brown cells signify positive cells in tumor tissues. (F) Statistical analysis, FXR1 (p = 0.003), N-cadherin (p = 0.008), E-cadherin (p = 0.0014), and TGF-β (p = 0.0001). Scale bars: 50 μm

Down-regulation of FXR1 attenuates the tumorigenesis of HCT116 cells

After transfection with shFXR1, HCT116 (shFXR1-HCT116) cells produced a smaller tumor than control cells (Figure 3 A - D). E-cadherin levels were considerably greater in tumor tissues produced from mice treated with shFXR1-HCT116 cells, which agreed with the findings of *in vitro* Western blot analysis. TGF-β and N-Cadherin levels, on the other hand, were considerably decreased (Figures 3 E and F). These results revealed that FXR1 knockdown significantly suppressed the tumorigenicity of HCT116 cells.

DISCUSSION

Fragile X-related protein-1 exhibits a crucial RNA-binding function, controls a variety of RNA functional abnormalities and is associated with a broad spectrum of human pathologies [10,11]. Furthermore, the 3'-UTRS-rich AU elements (AREs) in FXR1 have a strong regulatory ability after transcription, affecting the stability and translation of mRNA transcripts and contributing to differential clinical consequences [12,13]. FXR1 is expressed in multiple human organs, including the liver, brain and kidneys [14]. It is also crucial for proper embryonic and postnatal development of muscle and nervous system tissues [15,16]. Moreover, FXR1 has also been linked to a number of malignancies, including lung, intestinal, nasopharyngeal and kidney cancer, among others [17]. The oncogenic FXR1 overexpression in prostate cancer negatively impacted FBXO4 and loss of FBXO4 restores the tumor-suppressive phenotypes of FXR1-deficient cells [18].

Fragile X-related protein-1 was carcinogenic via MIR17HG/miR-346 and miR-425-5p/TAL1/DEC1 pathways [19]. It interacted with eukaryotic initiation factors (eIFs) to stabilize cMYC post-transcriptionally. The ovarian xenograft mouse model's total tumor growth, metastasis and survival were all decreased by stable FXR1 knockdown. These findings suggest that FXR1 is not only involved in the control of normal cellular physiology, but in cellular diseases as well, including tumor growth. In China during the past few decades, CRC incidence and mortality have both considerably increased. While early-stage patients can be treated effectively using surgery alongside radiotherapy and chemotherapy, nearly 90 % of patients experience poor prognoses due to recurrence. Further, the adverse effects of radiotherapy and chemotherapy are a persistent challenge in treating CRC. Therefore, effective biomarkers for early diagnosis and therapeutic intervention remain highly desirable [20,21].

Previous studies have confirmed that FXR1 was substantially expressed in CRC, but has not been verified by *in vivo* experiments and the mechanism underlying the biological behavior of FXR1 is unknown [8]. Therefore, the present study was carried out to investigate the function of FXR1 in CRC. First, FXR1 protein levels were determined relative to adjacent, non-cancerous tissue in human CRC tissues. The TCGA data was then used to analyze the correlation of FXR1 mRNA levels with differential prognosis among CRC patients. Finally, the use of RNA interference to knockdown the expression of FXR1 in HCT116 cells and explore the potential processes behind the biological role of FXR1 on HCT 116 *in vitro* along with *in vivo*, was assessed. The findings showed that FXR1 serves as a CRC promoter and FXR1 protein levels in human CRC tumor tissues were substantially greater than in surrounding tissues. The TCGA data analysis revealed that higher FXR1 mRNA levels correlated with poorer prognoses in CRC patients and FXR1 was a separate predictor of tumor differentiation and poor survival in CRC. Experimental knockdown of FXR1 expression within HCT116 cells significantly suppressed cellular proliferation, invasion, and tumorigenesis. Furthermore, the levels of N-cadherin was significantly reduced, while E-cadherin was increased. These findings are consistent with the conclusions of a recent investigation that showed FXR1 is linked to malignant biological activity of colorectal cancer, prostate cancer, and nasopharyngeal carcinoma [18,19,21].

Limitations of this study

Although FXR1 has been identified as a risk factor for transferring CRCs, the clinical significance and specific signal pathways of these changes are largely unknown and specific mechanisms need to be further clarified. FXR1, as an RNA-binding protein, is also critical in determining which regions interact with RNA.

CONCLUSION

The current study sheds new light on how FXR1 induces malignant biological activity in HCT116 cells through EMT. The findings show that an enhanced CRC therapeutic efficacy is attained by inhibiting FXR1 expression. As a result, FXR1 might be a potential therapeutic target in CRC.

DECLARATIONS

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None provided.

Ethical approval

None provided.

Availability of data and materials

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

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Qindan Du and Xiao Jian contributed equally to this work. Chen YQ should be considered as the co-corresponding author. Qindan Du, and Jiayao Chen conducted the experiments, YixinBian, Honglei Li and Ling Lin participated in the collection of tissue specimens for colon cancer, XS Ge analyzed the data, Xiaoying Wang and XS Ge wrote the manuscript, and Y Q Chen revised the manuscript. All authors reviewed and approved the final version of the manuscript for publication.

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