Original paper

Fat mass and obesity-associated protein expression in colorectal cancer and its influence on the biological behaviour of colorectal cancer cells

Huihui Wu[1], Yibo Wu[1], Zhishan Zhang[1], Qinglin Chen[1], Tingjin Zheng[1], Hui Zhong[2]

[1]Department of Clinincal Laboratory, Quanzhou First Hospital Affiliated to Fujian Medical University, Quanzhou, Fujian, China
[2]Clinical Laboratory, Fujian Maternity and Child Health Hospital, Fuzhou, Fujian, China

Fat mass and obesity-associated protein (FTO) was the earliest discovered m6A RNA demethylase. Previous studies have indicated that m6A modifications significantly influence the development, progression, and prognosis of various cancers. This study aimed to explore the role of FTO overexpression in colorectal cancer development, as well as its biological functions. Expression levels of FTO mRNA and protein in colorectal cancer and adjacent non-cancerous tissues were assessed using RT-PCR and immunohistochemistry. FTO overexpression was achieved by transiently transfecting an FTO overexpression plasmid into the HCT15 SW480 colorectal cancer cell line. The impact of this overexpression on the cells was evaluated using real-time fluorescence quantitative PCR, CCK8 proliferation assays, colony formation assays, scratch healing assays, and transwell migration and invasion assays. RT-PCR and immunohistochemistry demonstrated negligible or low FTO mRNA and protein expression in adjacent non-cancerous tissues, while high expression levels were observed in cancerous tissues. FTO overexpression in the HCT15 SW480 cell line significantly increased FTO mRNA levels compared to the control group. CCK8 assays indicated that cell proliferation was significantly higher in the FTO overexpressing group than in the control group. Colony formation assays revealed an increased number of colonies in the FTO group compared to controls. Scratch healing assays showed enhanced cell migration in the FTO group relative to controls. Transwell assays demonstrated a significant increase in invasive cell numbers in the FTO group compared to controls. In conclusion, FTO is highly expressed in colorectal cancer tissues, and its overexpression promotes proliferation and migration of colorectal cancer cells, underscoring its critical oncogenic role in this cancer type.

Key words: FTO, colorectal cancer, proliferation, migration, invasion.

Introduction

Fat mass and obesity-associated protein (FTO) was the earliest discovered m6A RNA demethylase. Previous studies have indicated that m6A modifications significantly influence the development, progression, and prognosis of various cancers [1]. Colorectal cancer (CRC) is a prevalent malignant tumour in the digestive system. In 2020, global CRC cases exceeded 1.9 million, making it the third most common cancer, and its mortality reached approximately 935,000, ranking second in cancer-related deaths.

In China, CRC accounts for 12.2% of cancer incidences, ranking second, and its mortality rate is 9.5%, ranking fifth in cancer-related deaths [2]. The rising incidence and mortality of CRC, exacerbated by improved living standards, changes in diet, and lifestyle, pose a significant public health challenge globally. Hence, understanding the molecular mechanisms of CRC development and progression is crucial.

Material and methods

Tissue samples

The study involved 36 CRC patients who underwent tumour resection at Quanzhou First Hospital Affiliated to Fujian Medical University from August 2019 to May 2022. Cancerous and adjacent non-cancerous tissues were extracted during surgery, immediately labelled, and stored in liquid nitrogen within 30 minutes. The non-cancerous tissues were taken from areas over 5 cm from the cancer lesions. Specimen collection received approval from the hospital's relevant departments and the Ethics Committee of Quanzhou First Hospital, with informed consent obtained from the patients or their families.

Reverse transcription polymerase chain reaction

To quantify FTO mRNA expression in CRC tissues, adjacent non-cancerous tissues, and various CRC cell lines (FHC, HCT, HF29, SW480), we employed reverse transcription polymerase chain reaction (RT-PCR). The reaction mixture for quantitative PCR (qPCR) comprised 2×qPCR SYBR Green Master Mix (10 μl), FTO-specific primers (0.4 μl each), 18s rRNA primers as an internal control (0.4 μl each), 50 × ROX Reference Dye II (0.4 μl), template cDNA (2 μl), and nuclease-free water to bring the total volume to 20 μl (Table I).

Immunohistochemistry

Tissue sections were initially baked at 60–65°C for 1–2 hours for deparaffinisation. Subsequent dewaxing and rehydration steps employed xylene and a graded series of ethanol. High-pressure and high-temperature antigen retrieval was performed, followed by quenching of endogenous peroxidase activity using 3% hydrogen peroxide for 15 minutes. After blocking non-specific binding with a serum block corresponding to the host species of the secondary antibody, the sections were incubated with primary antibodies overnight at 4°C. After primary antibody incubation, sections were washed and incubated with secondary antibodies at 37°C for 30 minutes. Colour development was achieved using DAB chromogen for 1–2 minutes, and counterstaining was performed with haematoxylin for 30–60 seconds.

Table I. RT-PCR primer sequences

PRIMER NAME	PRIMER SEQUENCE (5'--> 3')
FTO up	AATCTTGACTGCCATCCT
FTO down	CTGACCTCTGAGTTCTGAA
18s rRNA up	GTTCTTAGTTGGTGGAGCGATTTG
18s rRNA down	TTGCTCAATCTCGGGTGGC

FTO overexpression and quantitative PCR analysis

For transient transfection, HCT15 and SW480 CRC cell lines were incubated with either the FTO overexpression plasmid (pcDNA3.1-FTO) or control vector (pcDNA3.1). After 48 hours of incubation at 37°C in a 5% CO_2 atmosphere, total RNA was extracted and reverse-transcribed into cDNA. Quantitative analysis of FTO mRNA expression was then conducted using real-time fluorescence quantitative PCR (qPCR), with 18S RNA serving as an internal reference.

Cell counting Kit-8 (CCK8) proliferation assay

Cell proliferation was assessed using a CCK8 assay. Cells were plated in 96-well plates at a density of 2000 cells/100 μl per well, with 100 μl of PBS added to the outer wells to reduce evaporation. The plates were incubated at 37°C in a 5% CO_2 environment. At 0, 24, 48, and 72 hours post-seeding, 10 μl of CCK8 reagent was added to each well, followed by a further 2-hour incubation period.

Clonogenic formation assay

For the clonogenic assay, cells in the logarithmic growth phase were digested, resuspended in complete medium, and counted. Approximately 1000 cells were seeded per well in 6-well plates. The medium, supplemented with 10% FBS, was refreshed every 2 days. After 14 days of cultivation, the cells were fixed with 4% paraformaldehyde for 30 minutes and stained with Gentian violet for 30 minutes to visualise colonies.

Wound healing assay

In the wound healing assay, cell suspensions were plated in 6-well plates at a density of 2×10^6 cells per well and incubated overnight at 37°C in a 5% CO_2 incubator to allow for cell attachment and spreading. Uniform scratches were created using a 200 μl pipette tip, followed by treatment with complete medium containing 1 μg/mL mitomycin to inhibit cellular proliferation, thereby isolating the migratory response. Initial images were captured as a baseline (0 h).

Transwell invasion assay

The Transwell invasion assay was employed to examine the effects of FTO overexpression on the invasive capabilities of CRC cells. This assay facilitates the evaluation of cell migration through a porous membrane, simulating the invasion through the extracellular matrix.

Statistical analysis

All results were obtained from at least 3 independent experiments, and data were presented as the mean ±SD. For comparisons of 2 groups, 2-tailed paired Student's *t*-tests were conducted.

Results

mRNA expression levels of FTO in colorectal cancer tissues and cell lines

Quantitative RT-PCR was employed to assess the mRNA expression levels of FTO in CRC tissues and adjacent non-cancerous tissues from the same patients (Fig. 1A). Additionally, FTO mRNA expression was evaluated in various human CRC cell lines (HCT, HF29, SW480) compared with normal intestinal epithelial cell lines (FHC), as depicted in Figure 1B.

Protein expression levels of FTO in colorectal cancer tissues

In the study of 36 CRC patient groups, the FTO protein's expression levels in cancerous and adjacent tissues were semi-quantitatively assessed using immunohistochemistry. The results of the staining were examined under an inverted microscope. As depicted in Figure 2A, the FTO protein was primarily found in the glandular cytoplasm within the cancer tissue.

The Soslow scoring system was employed, in which the staining intensity of positive cells was scored as 0, 1, 2, and 3 points corresponding to no staining, light yellow, brown, and dark brown, respectively. The rate of positive cells was determined by taking the average of 5 positive cell counts and scored as 0, 1, 2, 3, and 4 points corresponding to $< 5\%$, $5\% \sim 25\%$, $25\% \sim 50\%$, $51\% \sim 75\%$, and $> 75\%$, respectively. The product of these 2 scores resulted in the final score: 0 was classified as negative (–), $1 \sim 2$ as weak positive (+), $3 \sim 4$ as positive (++), and anything greater than 4 as strong positive (+++).

Upon scoring and analysing the staining results of each group, it was observed that the FTO protein was either not expressed or expressed at low levels in the tissues adjacent to the cancer. However, it was highly expressed in the cancerous tissues (Fig. 2B) ($p < 0.05$).

FTO expression in HCT15 and SW480 cell lines post-transfection

After transfection with pcDNA3.1-FTO, a significant upregulation of FTO mRNA was observed in HCT15 and SW480 cells, as shown in Figure 3, indicating successful overexpression of FTO.

Impact of FTO overexpression on colorectal cancer cell proliferation

The CCK8 proliferation assay was used to evaluate the impact of FTO overexpression on CRC cell proliferation. At time intervals of 0, 24, 48, and 72 hours post-incubation, the OD values at 450 nm were measured following the addition of

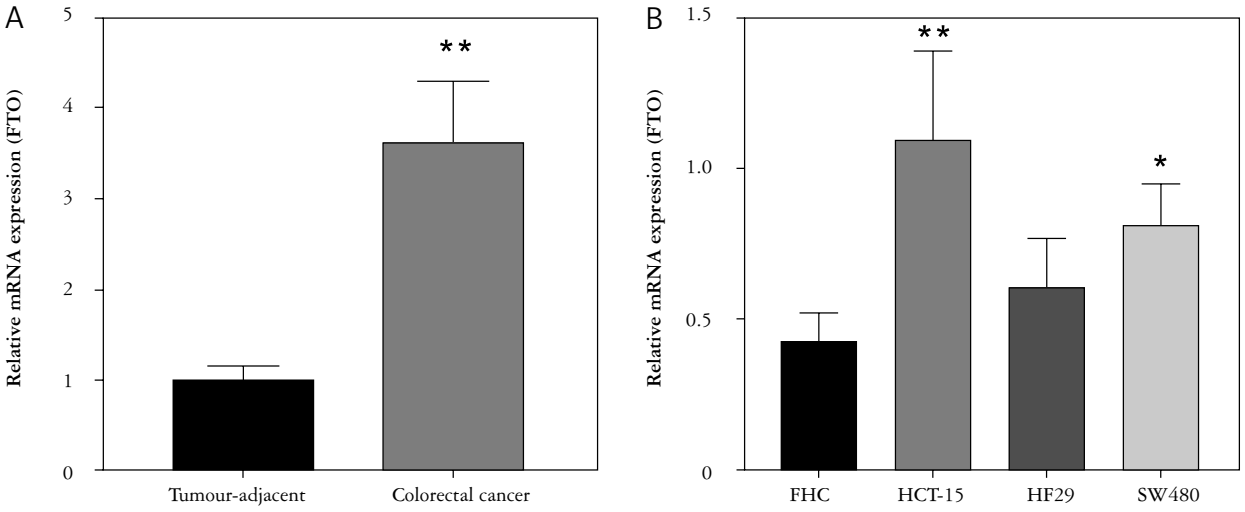
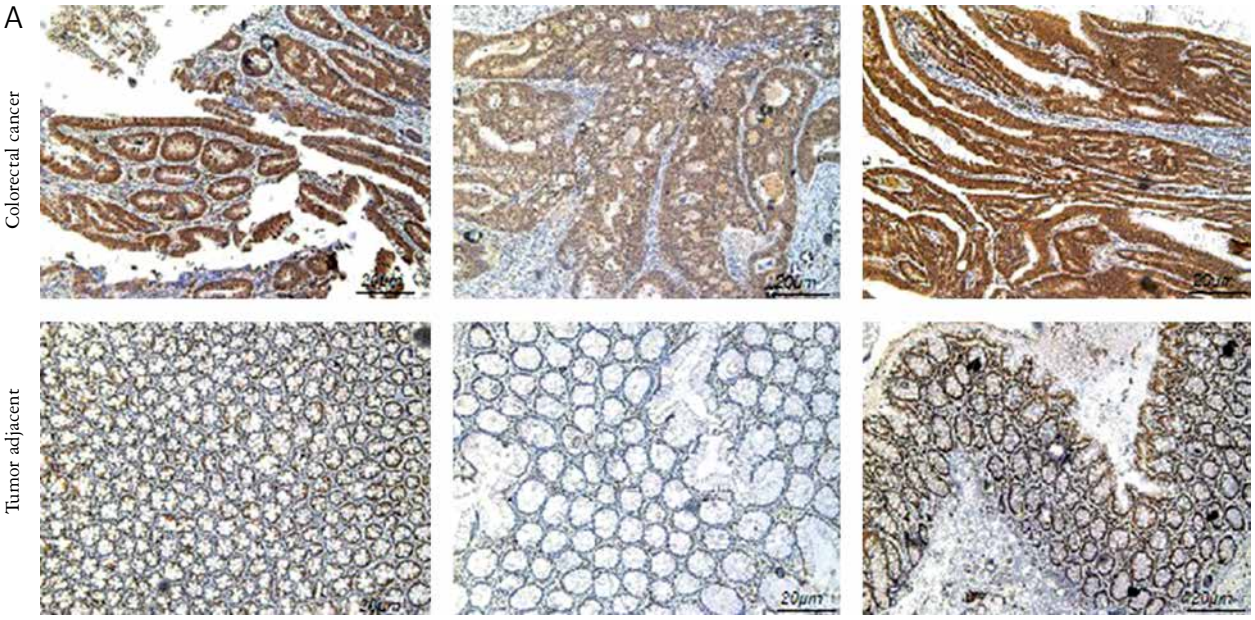


Fig. 1. A) Analysis of fat mass and obesity-associated protein (FTO) mRNA in colorectal cancer tissues and their adjacent non-cancerous tissues using quantitative RT-PCR. **B**) Expression levels of FTO mRNA in normal intestinal epithelial cell lines (FHC) and various human colorectal cancer cell lines (HCT, HF29, SW480) ($n = 3$, when compared with tumour-adjacent tissues or FHC cells, $*p < 0.05$; $**p < 0.01$)



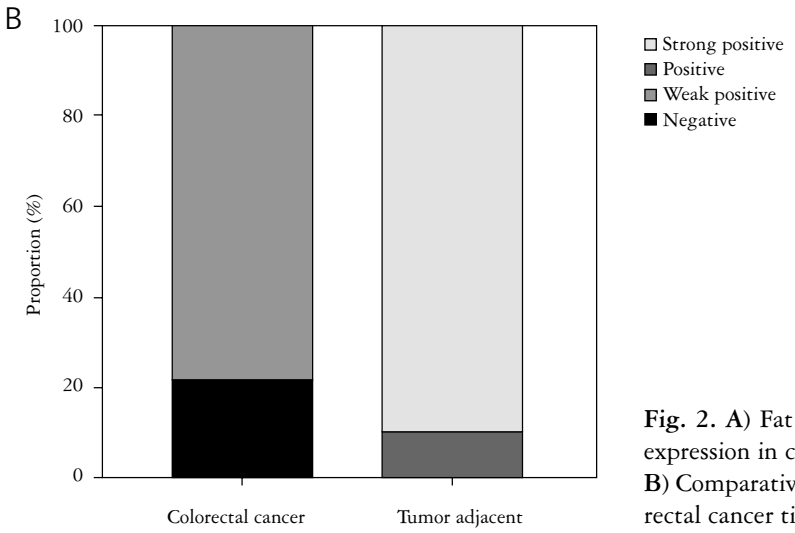


Fig. 2. A) Fat mass and obesity-associated protein (FTO) expression in colorectal cancer tissues and adjacent tissues. **B**) Comparative analysis of FTO protein expression in colorectal cancer tissues and adjacent tissues across 36 groups

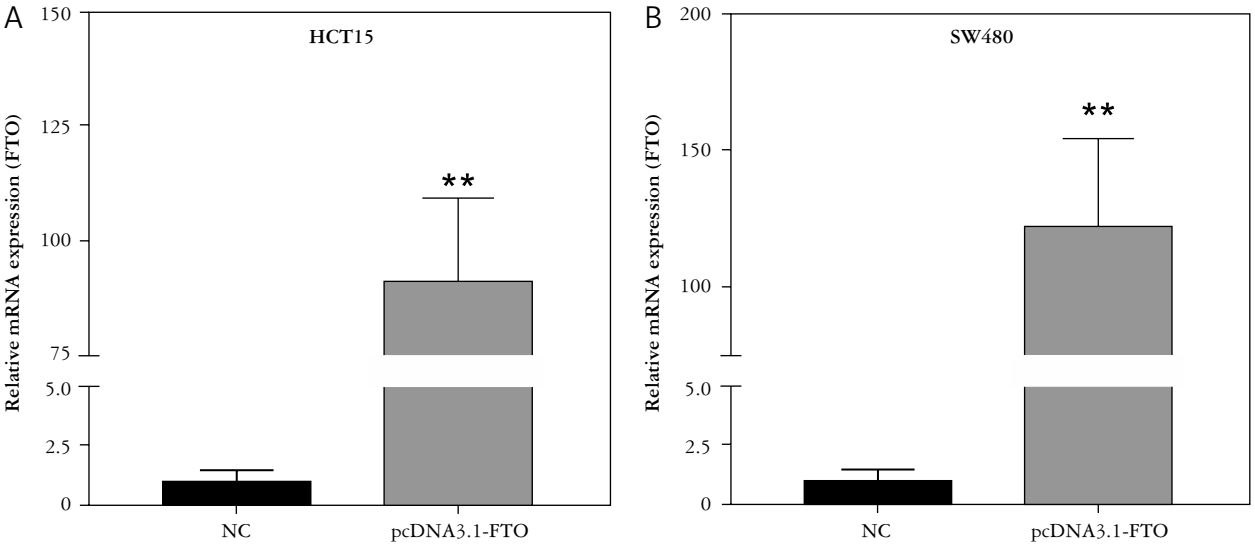


Fig. 3. Fat mass and obesity-associated protein (FTO) mRNA levels in HCT15 (**A**) and SW480 (**B**) cells after transfection with pcDNA3.1-FTO (**$p < 0.01$)

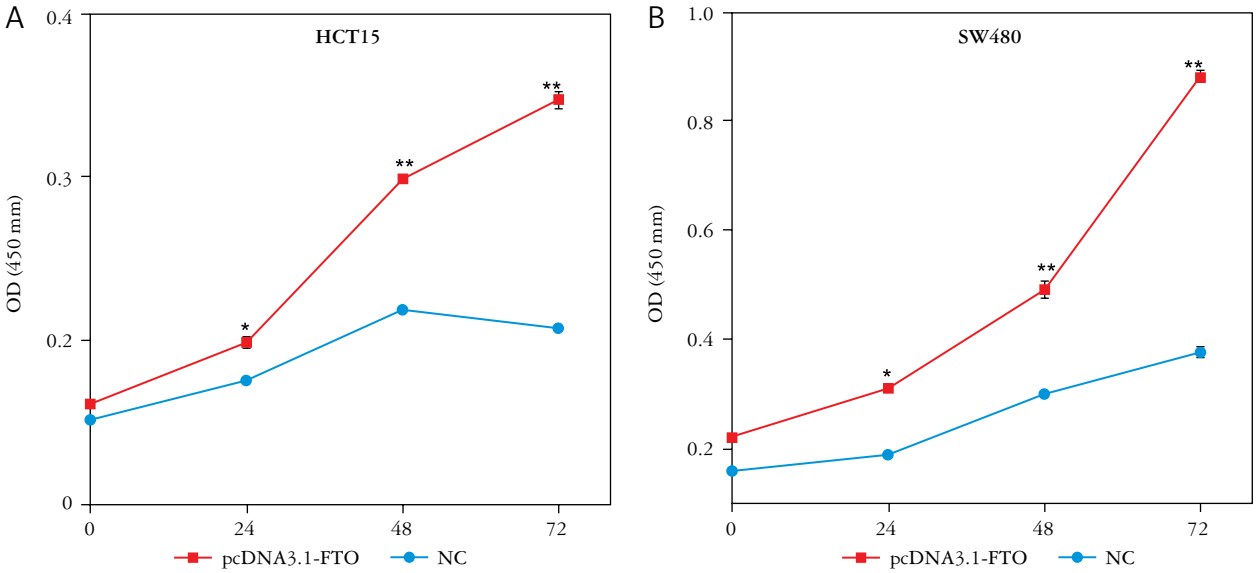


Fig. 4. The influence of fat mass and obesity-associated protein overexpression on the proliferation of HCT15 (**A**) and SW480 (**B**) cells (*$p < 0.05$; **$p < 0.01$)

10 μl CCK8 reagent and a subsequent 2-hour incubation. The results indicated a significant enhancement in cell proliferation in the FTO overexpressed group compared to the control (NC) group (Fig. 4).

Effect of FTO on clonogenic capability of colorectal cancer cells

Post-cloning in 6-well plates, cells were fixed, stained, and analysed for clonal colony number using ImageJ software. The FTO overexpressed group demonstrated a significantly higher number of clonal colonies compared to the control group, suggesting that FTO overexpression enhances the clonogenic potential of CRC cells (Fig. 5).

FTO promotes cell migration in colorectal cancer cell lines

The migration ability of cells was quantified using a scratch healing assay. ImageJ software was employed to calculate the healing rate and percentage of wound closure over time. Compared to the control group, the FTO overexpressed group exhibited a significantly increased healing rate at 24 hours, indicative of enhanced migratory capacity ($p < 0.05$) (Fig. 6).

FTO augments invasion capability of colorectal cancer cells

The invasion potential was assessed using a Transwell assay. Cells were fixed with formaldehyde, stained with crystal violet, and counted under a microscope across multiple fields. The mean value was used for statistical analysis. A significant enhancement in the invasion capability was observed in the FTO overexpressed group compared to the control group ($p < 0.01$) (Fig. 7).

Discussion

In this study, RT-PCR and immunohistochemistry were used to conduct a semi-quantitative analysis of FTO mRNA and protein in 36 groups of CRC tissues and corresponding adjacent tissues, revealing a significantly higher expression level of FTO in CRC cancer tissues compared to normal tissues. The effects of FTO overexpression on the biological function of CRC cells were investigated *in vitro*, showing that FTO overexpression promoted CRC cell proliferation, as shown by CCK8 proliferation and clonal formation experiments. Additionally, FTO overexpression significantly enhanced cancer cell migration and invasion, as confirmed by cell scratch healing and invasion experiments.

Existing research evidence indicates that FTO with m6A demethylation modification is highly expressed in various malignant tumours. Dysregulation of FTO has been associated with decreased m6A methylation levels in pancreatic cancer, thereby promoting cancer cell proliferation through the induction of PDGFC autocrine activity, which correlates with poor prognosis [3]. In studies related to gastric cancer, high FTO expression has been linked to increased proliferation, migration, and invasion of gastric cancer cells by down-regulating the m6A level of ITGB1 mRNA, thereby enhancing ITGB1 expression and impacting patient survival rates [4]. Additionally, FTO up-regulation in gastric cancer liver metastasis tissues has been found to promote caveolin-1 mRNA degradation, influencing mitochondrial fission and metabolism [5]. These findings underscore the diverse targets and mechanisms through which FTO plays a role in cancer development.

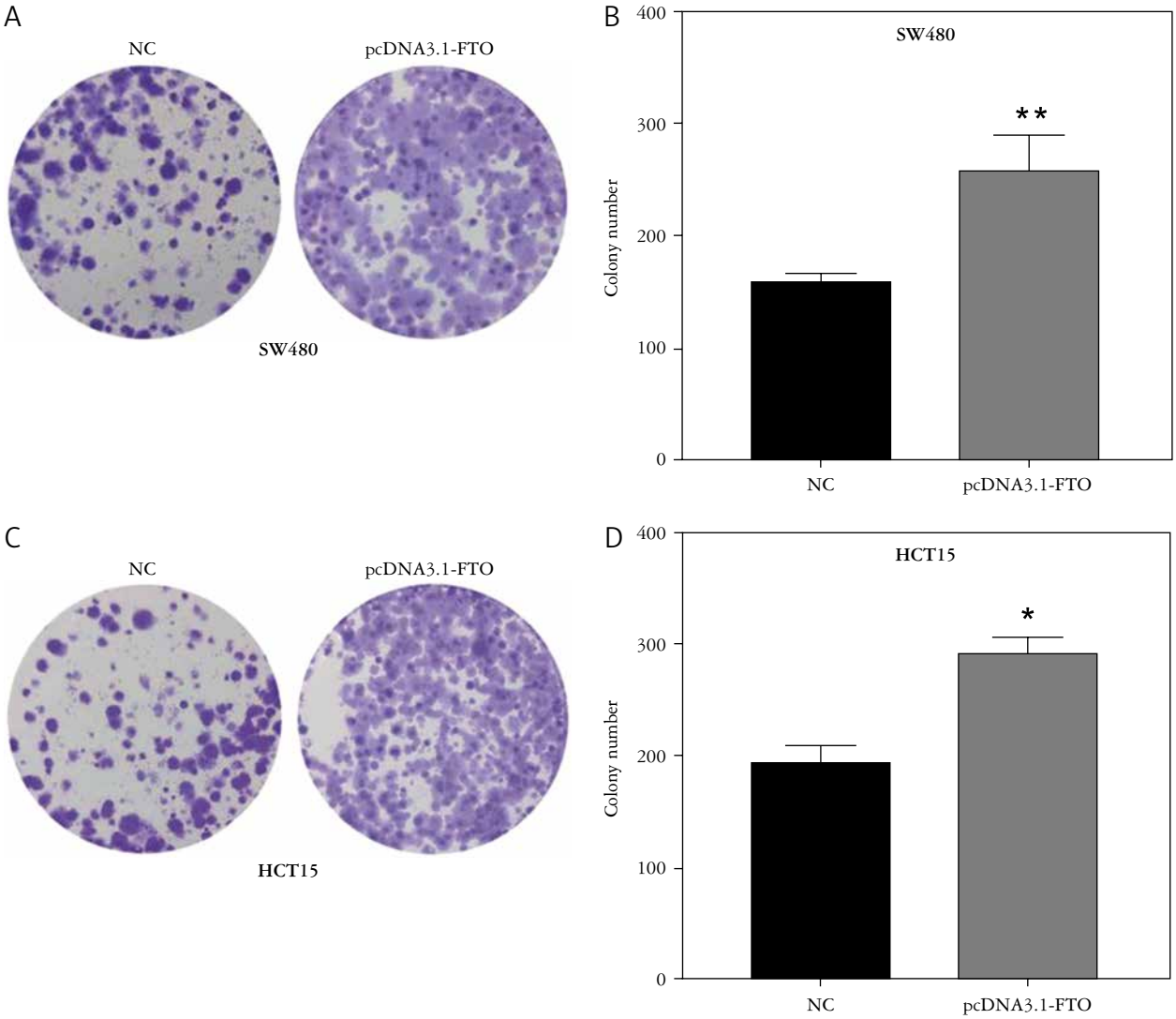


Fig. 5. Fat mass and obesity-associated protein overexpression and its effect on clonal formation in HCT15 and SW480 cells. **A, C**) Cell clonal formation results. **B, D**) Statistical chart of colony number of cell clones (*$p < 0.05$; **$p < 0.01$)

Studies on oesophageal squamous cell carcinoma (ESCC) suggest that FTO promotes ESCC cell proliferation and migration, primarily through MMP13 up-regulation, with poor patient prognosis closely associated with FTO overexpression [6]. FTO is also highly expressed in other malignant tumours such as endometrial cancer, melanoma, and non-small cell lung cancer [7–9]. Its mechanism involves the regulation of downstream target genes' m6A methylation through FTO's demethylase activity, thereby contributing to tumour occurrence, migration, and invasion.

Interestingly, the role of FTO protein in tumour development is multifaceted, demonstrating bidirectional regulatory effects across different cancer types. Studies highlight FTO as a tumour suppressor in ovarian cancer, inhibiting ovarian cancer stem cells' self-renewal, proliferation, and tumour initiation [10]. Similarly, in thyroid papillary carcinoma, FTO inhibits tumour growth and proliferation by modulating m6A modification of APOE, which impacts glycolytic metabolism [11].

In CRC, FTO is up-regulated and can activate MYC by reducing m6A modification, thereby stimulating CRC cell proliferation and invasion [12]. This mechanism mirrors the interaction observed in cervical cancer, further emphasising FTO's oncogenic role in CRC [13]. Our study confirms this, concluding that FTO overexpression in CRC mediates cancer cell proliferation and migration, suggesting its oncogenic function in CRC. This finding aligns with research in colon cancer, which identified a pathway in which FTO influences glycolysis through PKM2, affecting tumour vitality [14].

However, a study by Ruan DY found significantly down-regulated FTO protein levels in CRC tissues, correlating high FTO expression with favourable patient prognosis [15]. This discrepancy underscores the need for further investigation, considering factors like sample size and staging. Moreover, the com-

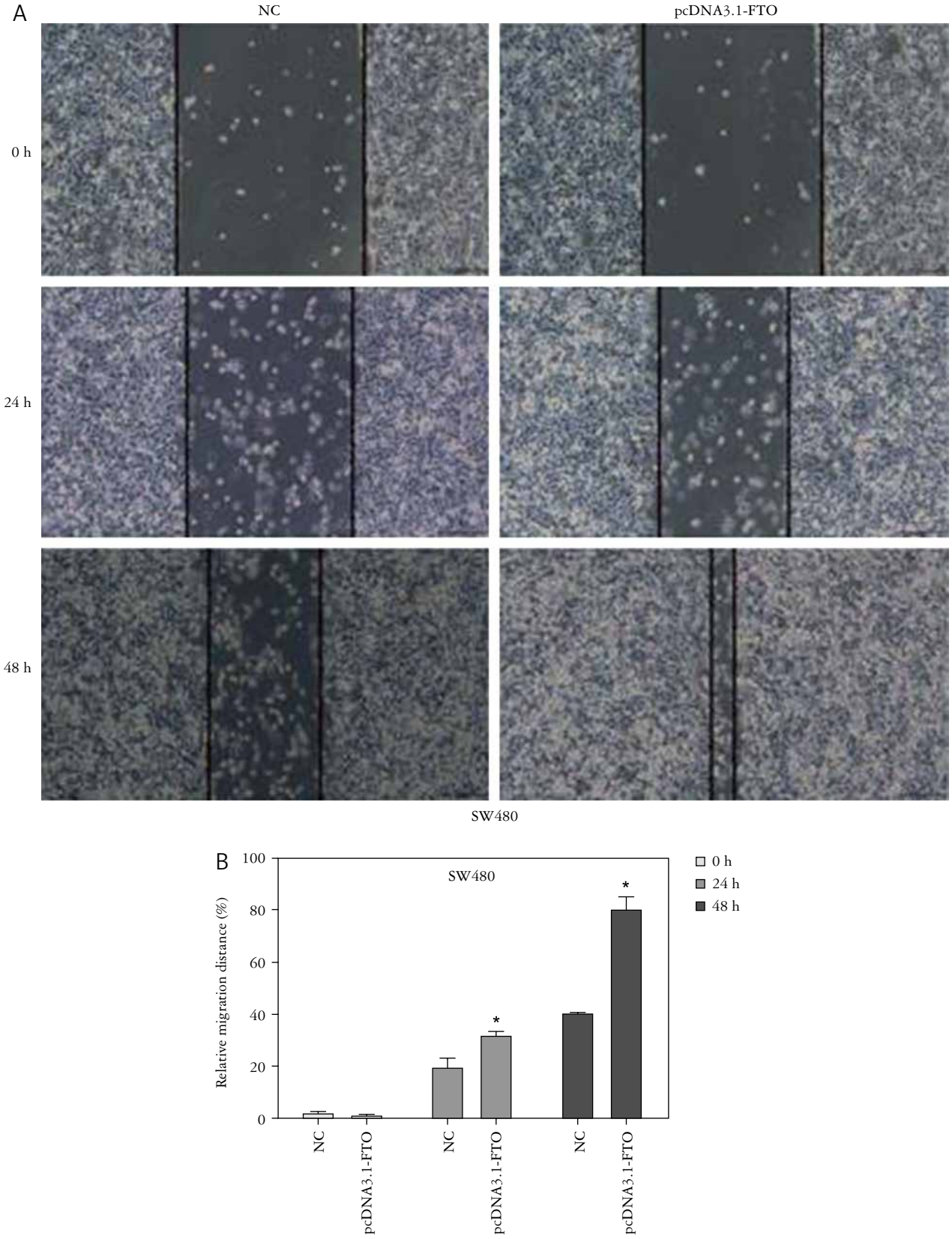
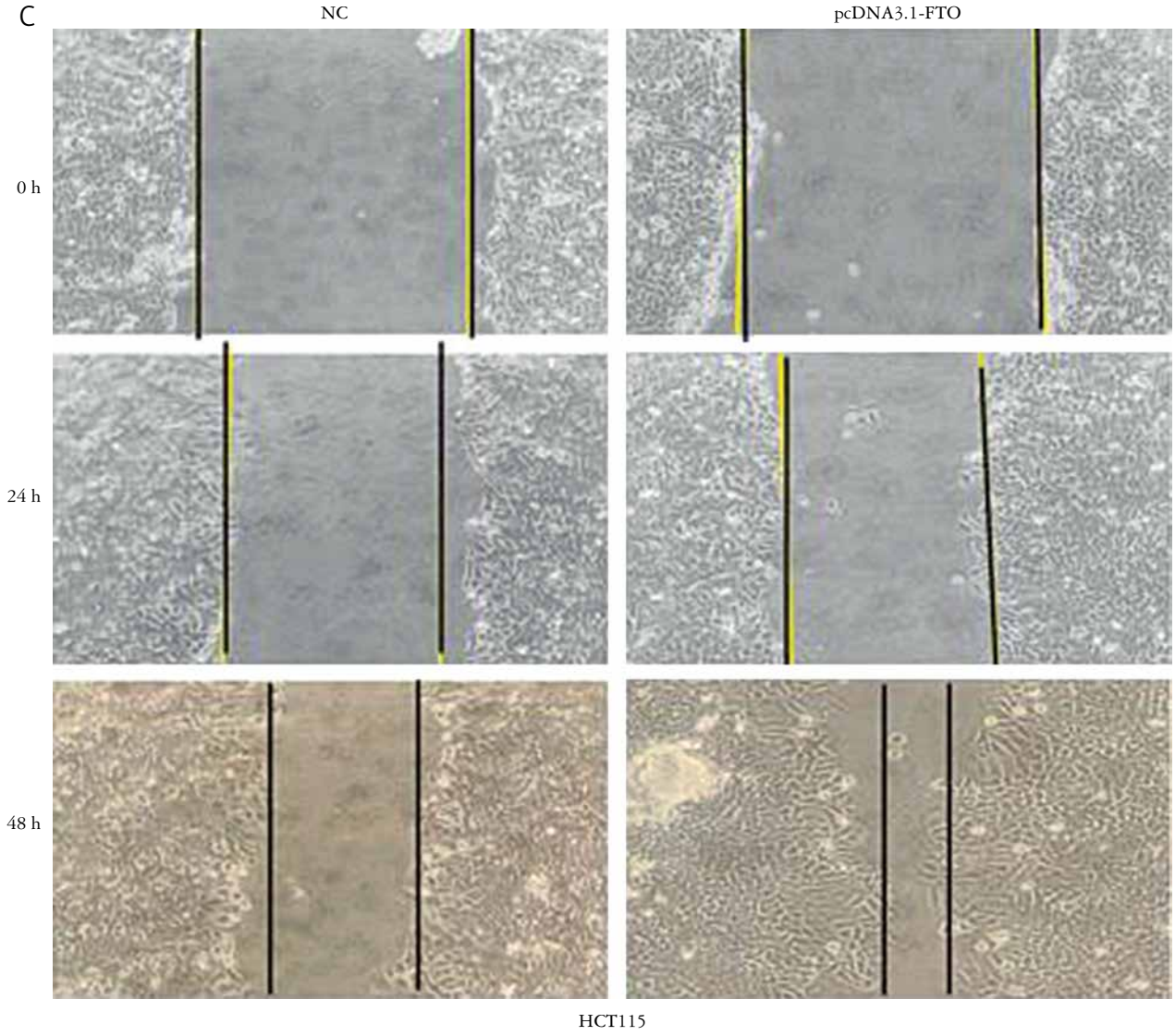


Fig. 6. The effect of FTO overexpression on the migration of HCT15 and SW480 cells. **A, C**) Cell scratch healing results. **B, D**) Statistical chart of cell scratch healing rate (*$p < 0.05$)


C
NC
pcDNA3.1-FTO
0 h
24 h
48 h
HCT115
D
HCT115
Relative migration distance (%)
100
80
60
40
20
0
0 h
24 h
48 h
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NC
pcDNA3.1-FTO
NC
pcDNA3.1-FTO
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Fig. 6. Cont.

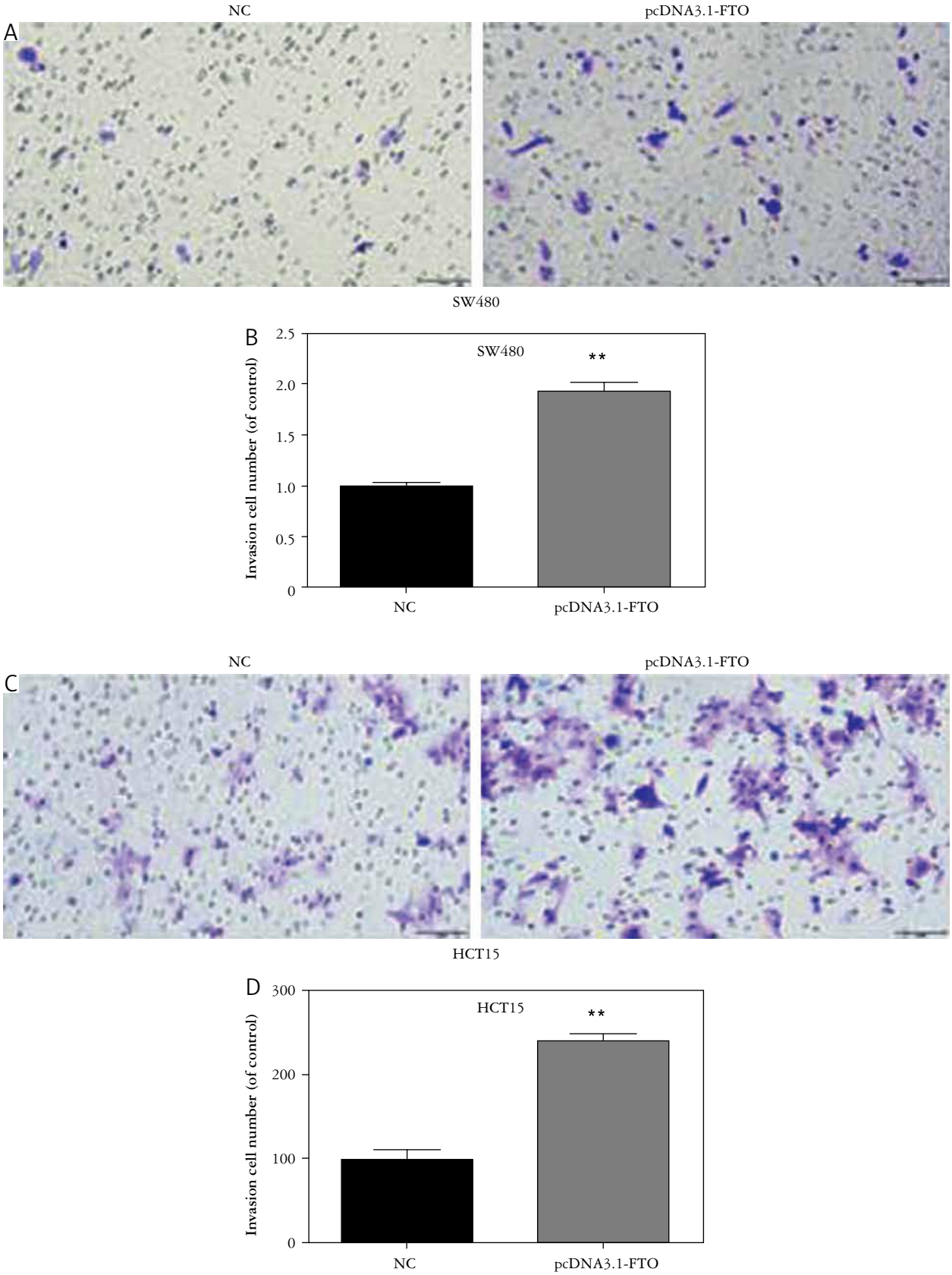

Fig. 7. Influence of FTO overexpression on the invasion capacity of HCT15 and SW480 cells. **A, C**) Transwell results. **B, D**) statistical chart of cell invasion rate (**$p < 0.01$)

plexity of m6A modification and its downstream effects necessitates comprehensive research to elucidate FTO's role in CRC. Future studies should include larger sample sizes, establish knockdown FTO cell lines, and investigate downstream target genes and related pathways to enhance our understanding of FTO's influence on CRC biology.

We observed a significant up-regulation of FTO expression in CRC, promoting CRC cell proliferation, migration, and invasion. Inhibiting FTO expression may offer therapeutic potential in CRC treatment, although its specific mechanisms require further elucidation.

Disclosures

1. The study received approval from the Ethics Committee of Quanzhou First Hospital, approval number: 2020-253.
2. Assistance with the article: None.
3. Financial support and sponsorship: Funding for this study was provided from an intramural grant by Quanzhou Science and Technology Program Project, China (Project number: 2020N021S, Guideline code: 2020N003).
4. Conflicts of interest: None.

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Address for correspondence:

Tingjin Zheng
Department of Clinical Laboratory
Quanzhou First Hospital Affiliated to Fujian Medical University
248-252 Dong Road
Quanzhou, China
e-mail: 15060810826@163.com

Hui Zhong
Clinical Laboratory
Fujian Maternity and Child Health Hospital
18 Daoshan Road
Gulou District, Fuzhou, China
e-mail: 122397355@qq.com