LncRNA LEF1-AS1/LEF1/FUT8 Axis Mediates Colorectal Cancer Progression by Regulating α 1, 6-Fucosylation via Wnt/ β -Catenin Pathway

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Abstract

Background: Fucosylation alteration is involved in several steps of human cancer pathogenesis. Dysregulated long noncoding RNA (lncRNA) often leads to malignancy in colorectal cancer (CRC).

Methods: Differential levels of LEF1-AS1, LEF1 and FUT8 are analyzed by qRT-PCR and western blot. Chip, RIP, EMSA and luciferase reporter assay confirm the direct interaction among LEF1-AS1, MLL1, H3K4me3, LEF1 and FUT8. Functionally, CRC cell proliferation, proliferation, migration and invasion are analyzed by CCK8 assay, colony formation assay, transwell assay and flow cytometry. The xenografts nude mice models, lung metastasis and liver metastasis are established to determine the effect of LEF1-AS1/LEF1/FUT8 axis on CRC progression *in vivo*.

Results: Here, we identify that LEF1-AS1 and LEF1 are higher in CRC tissues than that in adjacent tissues, as well as upregulated in CRC cell lines than that in normal colorectal cells. Altered levels of LEF1-AS1 modulate LEF1 expression, while altered LEF1 could not regulate LEF1-AS1. LEF1-AS1 recruits MLL1 to the promoter region of LEF1, induces H3K4me3 methylation modification and mediates LEF1 transcription. Furthermore, α 1-6 fucosyltransferase FUT8 is overexpressed in CRC tissues and positively correlated to LEF1. FUT8 is a direct target of transcription factor LEF1, which regulates FUT8 level. Altered FUT8 also regulates the core fucosylation of CRC cells, and LEF1-AS1 mediates FUT8 level through activation of Wnt/ β -catenin/LEF1 pathway, thereby resulting in β -catenin nuclear translocation. In addition, LEF1-AS1 mediates the proliferation, migration and invasion of CRC cells *in vitro*. LEF1-AS1 silence hinders the tumorigenesis, liver and lung metastasis of SW620 cells *in vivo*, while overexpressed FUT8 abolishes the suppressive impact of LEF1-AS1 repression on the biological behavior of SW620 cells.

Conclusion: Our studies uncovered a novel mechanism for constitutive LEF1-AS1/LEF1/FUT8 axis in CRC progression by regulating α 1, 6-fucosylation via Wnt/ β -catenin pathway, and consequently, as a potential therapeutic target in CRC.

Background

Colorectal cancer (CRC) is the third most common cancer in the world [1]. Distant metastasis is a leading cause of cancer-related death in CRC patients [2]. The early stage of non-metastatic CRC can be treated with surgery followed by chemotherapy or radiotherapy. The 5-year survival rate is higher than 90% [3]. Most CRC patients with liver metastases are not suitable for surgery, in consequence, the 5-year survival rate of these patients is less than 10% [4]. Therefore, it is critical to identify the specific metastasis biomarkers for CRC patients.

Long non-coding RNAs (lncRNAs) are a subgroup of non-coding RNAs greater than 200 nucleotides in length. lncRNA may provide clinical information on tumor outcomes and be used as a prognostic marker [5]. Lymphoid enhancer-binding factor 1 (LEF1) antisense RNA 1 (LEF1-AS1), is a highly conserved transcript and a newfound lncRNA encode in the plus strand of LEF1 at chromosome 4q25, which is

firstly found to be upregulated and predict prognosis in CRC[6]. In recent years, the distinct upregulation of LEF1-AS1 was demonstrated in several tumors, such as glioblastoma, oral squamous cell carcinoma, lung cancer and prostate cancer[7–10]. However, LEF1-AS1 is highly expressed in normal hematopoietic stem cells but barely detectable in myeloid malignant cell lines [11]. Even though the biological function of LEF1-AS1 has been analyzed in different cancers, the regulatory pattern of LEF1-AS1/LEF1/FUT8 in CRC development via core fucosylation is not revealed.

Accumulating evidence suggests that changes in glycosylation patterns are associated with cancer development. Altered glycosylation primarily occur from changes in the expression of glycosyltransferases (GTs) [5]. Fucosylation, one of the most common types of glycosylation, is regulated by fucosyltransferases (FUTs). Deficiency or overexpression of FUTs has been reported in many cancers, and contribute to the malignant phenotype[12, 13]. Agrawal et al. reveal that FUT8 mediates core fucosylation and alters L1CAM proteolytic cleavage and invasion of melanoma cells[14]. In breast cancer, the upregulation of FUT8 and α 1, 6-fucosylation induce stemness and EMT by activating Wnt/ β -catenin signaling pathway [15]. Our previous study indicates that FUT8 expression is significantly up-regulate in high metastatic hepatocellular carcinoma cells and tumor tissues[16]. However, it is still urgent to clarify the mechanism of FUT8 involved in core fucosylation during CRC malignancy.

Abnormal activation of the Wnt/ β -catenin pathway often occurs in carcinogenesis, most notably CRC where a hyperactive Wnt/ β -Catenin pathway drives tumour cell proliferation[17]. Mutations in Wnt/ β -catenin pathway components including APC, Axin, and β -catenin itself are well-established causes of aberrant signaling activation leading to cancer. These genetic defects lead to the accumulation of β -catenin in the nucleus[18]. β -catenin shuttles between the cytosol and the nucleus, where it acts as a cotranscription factor to induce expression of proto-oncogenes such as c-Myc and cyclin D1. Blocking Wnt/ β -catenin signaling in CRC cells can induce apoptosis or growth inhibition[19]. LEF1, a member of the T-cell Factor (TCF)/LEF1 family of high-mobility group transcription factors, is primarily involved in the canonical Wnt/ β -catenin signaling pathway[20]. Wnt/ β -catenin signaling pathway mediates the upregulated FUT8 and α 1, 6- fucosylation level, which contributes to the boost of breast cancer stemness[15].

In the current study, we revealed a novel mechanism that LEF1-AS1/LEF1/FUT8 axis could act as a driver in CRC progression via fucosylation and Wnt/ β -catenin pathway, and might provide a novel strategy for the treatment of CRC patients.

Materials And Methods

Clinical specimen and cell lines

Human primary CRC and adjacent tissues were extracted from 36 patients who underwent surgical resections from the First Affiliated Hospital of Dalian Medical University. All tumor tissues were confirmed by histological examination as adenocarcinoma of the colorectal. Tissues were stored in liquid nitrogen for Quantitative Real-Time PCR (QRT-PCR) and immunohistochemistry experiments. The research

protocol was approved by the Ethics Committee of the First Affiliated Hospital of Dalian Medical University (YJ-KY-FB-2016-16). CRC cell lines HCT-8, Caco2, SW480, SW620, LOVO and human normal colorectal epithelial cell line (FHC) were obtained from KeyGEN Company (Nanjing, Jiangsu, China) and maintained as previously described[21].

Cell transfection

The amplifications of LEF1-AS1 and FUT8 were clone into the expression vector pcDNA3.1 (Invitrogen). Small interfering RNA of LEF1-AS1, LEF1 and FUT8, scramble siRNA (siSCR), short hairpin RNA for LEF1-AS1 and scramble shRNA were synthesized by GenePharma. Lipofectamine 3000 Reagents (Invitrogen Co., Carlsbad, CA, USA) were used for cell transfection. QRT-PCR was used to assess the transfection efficiency.

RNA isolation and qRT-PCR

Total RNA extraction, from the clinical tissues and cell lines was accomplished by TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and using the QuantiTect Reverse Transcription Kit (QIAGEN, Valencia, CA, USA) for cDNA synthesized according to the manufacturer's protocol. RT-PCR analyses were performed on LightCycler 96 detection system (Roche) according to the manufacturer's instructions. All mRNA expression levels were normalized to GAPDH. Relative fold differences were determined using the method of delta–delta CT.

Western blot analysis

Cells were washed with phosphate-buffered saline (PBS) and dissolved in a lysis buffer containing a mixture of protease inhibitors, and the total protein was obtained. Proteins were electrophoresed with 10% SDS-PAGE gels and transferred to the polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Primary antibodies were incubated at 4°C overnight. Membrane proteins were incubated with anti-rabbit IgG at 37 °C. Immunodetection was acquired with the ECL Western blotting kit. All bands were analyzed by LabWorks (TM ver4.6, UVP, BioImaging Systems, NY, USA).

Lectin blot analysis

The CRC cells were lysed and separated via 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Then the PVDF membranes were incubated with the biotinylated Lens Culinaris Agglutinin (LCA) (Vector Laboratories, Burlingame, CA, USA) for 1h at room temperature. The membranes were subsequently incubated with horseradish peroxidase streptavidin (Vector Laboratories) for 30 min and detected with an ECL Western blot kit.

Cell growth curve

To assess proliferation, cells were grown for 48 hours (h) under various experimental conditions and proliferation was determined by using the Cell Counting Kit-8 (CCK-8) assay according to the

manufacturer's instructions. 1×10^3 /well density cells were seeded in 96-well plates and then cultured for 0, 24, 48, 72 h. At these different stages, CCK-8 reagent was added to each well. The optical density (OD) at 450 nm was read in a microplate reader. (Bio-Rad, Hercules, CA, USA)

Colony formation assays

Cells were plated in six well plates at 1.5×10^3 per well. The medium was renewed at regular intervals. After incubated for 12 days, the colonies were fixed by 4% paraformaldehyde for 20 min, and stained with 0.2% crystal violet. Colonies were photographed and analyzed.

5-Ethynyl-20-deoxyuridine (EDU) incorporation assays

The EDU assay was performed with a KFluor488 EDU Kit (KeyGEN BioTECH, Nanjing, China). Equal number cells were inoculated in 96-well plate overnight. After treated with 50 μ M EDU for 2 h at 37 °C, the cells were fixed with 10% formaldehyde, and stained with Click-iT reaction mixture and Hoechst 33342. Images were captured with microscope.

Invasion and wound healing assays

Boyden transwell chambers (Corning, New York, USA) were used to detect cell invasive ability in vitro. Invasion assay was performed as described previously[21]. The number of invaded cells on each membrane was counted from 5 random fields using an inverted microscope (20 $\times$ 10).

Cell migration potential was evaluated by measuring the movement of cells that was prepared as described previously[21]. Wound closure was photographed at 0 and 24h after wounding. The migration capability was quantified by the relative gap distance. The experimental results were analyzed with the software ip6.0.

Flow cytometry analysis

CRC cells with corresponding treatment were collected (5×10^5). After incubation with 5% BSA, the cells were incubated with FITC-LCA (Vector Laboratories Inc., Burlingame, CA, USA) for 60 min. CRC cells were resuspended in PBS. The FITC fluorescence intensity was immediately detected by FACS Calibur (Becton-Dickinson, CA, USA).

RNA immunoprecipitation (RIP) assay

RIP assay was performed using the Magna RIP™ RNA Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA, USA). CRC cells were collected and lysed in RIP lysis buffer containing a protease inhibitor cocktail and RNase inhibitor. The cell extracts were incubated with RIP buffer containing magnetic beads conjugated with human anti-Ago2 anti-body (Millipore) or negative control IgG overnight at 4°C. The protein was digested with proteinase K, total RNAs was isolated from the extracts using the TRIzol reagent subsequently. Purified RNA was analyzed by qRT-PCR.

Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed using the ChIP chromatin immunoprecipitation kit (Beyotime, Shanghai, China). Immunoselections of cross-linked protein-DNA were performed using MLL1 or anti-H3K4me3 antibody or rabbit non-immune IgG (as negative control) overnight at 4°C. The precipitated DNAs were analyzed by PCR.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed according to the manufacturer's instructions. The biotin 3' end DNA labeling kit and the LightShift chemiluminescent EMSA kit from Pierce Biotechnology were used in the test. DNA probe for EMSA was synthesized as oligonucleotides (the sequence: ATTAACCTTGATCTAGC). In brief, nuclear extracts were isolated from cells and performed with immunoprecipitation by LEF-1 antibody (abcam, UK). The protein extracts were incubated biotin-labeled probes for 20 mins. Then bound DNA complexes were separated on a 5% nondenaturing polyacrylamide gel electrophoresis and transferred to a nylon membrane (Roche). Finally, Biotin-labeled DNA-protein complexes were detected by the streptavidin conjugated with HRP.

Luciferase reporter assay

The pGL3-LEF1 promoter reporters were cotransfected into SW480 and SW620 cells with siSCR or siLEF1-AS1. After 48 hours, the luciferase activity of pGL3-LEF1 promoter was tested by the Luciferase reporter assay system. TOP-Flash and FOP-Flash reporters were cotransfected into SW480 and SW620 cells with siSCR or siLEF1-AS1. At 48 h after transfection, cells were harvested and the relative activities of luciferase were monitored by means of luciferase reporter assay system.

Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde for 20 min and then permeabilized with 0.2% Triton X-100 for 5 min. After permeabilization, cells were incubated with 5% BSA for 30 min and incubated with primary antibodies overnight at 4°C. Cells were incubated with secondary antibody for 1h and then incubated with 4,6-diamino-2-phenylindole (DAPI, Solarbio, Beijing, China) for 5 min. Images were obtained using a fluorescence microscope.

Immunohistochemistry(IHC) staining

The tissue samples were collected and embedded with paraffin. A 5µm thick slice was cut and then tissue slices were deparaffinized, rehydrated, and immersed in 3% hydrogen peroxide for 10 min to block the endogenous peroxidase. The slices were incubated with the corresponding primary antibodies overnight at 4°C. Next day, the secondary streptavidin-horseradish peroxidase-conjugated antibody staining was performed at room temperature, visualized in 3, 3'-diaminobenzidine (DAB) (ZLI9018, ZSGBBIO, China), then counterstained with hematoxylin, dehydrated and cover-slipped.

Animal models

The male nude mice (4-5 weeks old) were purchased from the Model Animal Research Institute of Nanjing University. 2×10^6 CRC cells in 0.2 ml PBS were subcutaneously injected into the right armpit region of 25 male BALB/c nude mice which were randomly divided into five groups ($n=5$ for each group). After the seventh day, the tumor size was measured every 5 days with a caliper. Twenty-seven days after the injection, the mice were sacrificed and the tumors were collected and measured.

For the liver metastasis model, the spleen was exposed and injected with 2×10^6 CRC cells slowly, this procession sustained at least 5 min. Carefully sutured the wound and kept the nude mouse warm and in a sterile environment after surgery. After 6 weeks, the mice were sacrificed, and their spleens and livers were dissected out and removed for IVIS (Carestream Health, Inc.). The lung metastasis model was established by injecting 2×10^6 CRC cells into the tail vein. After 4 weeks, the mice were sacrificed, tumor in lung metastasis was removed for IVIS (Carestream Health, Inc.).

Statistical analysis

Each experiment was performed at least in triplicate. Data are presented as means $\pm$ SD and were analyzed using SPSS 17.0. Student's t-test was used to compare the means of two groups. The significance of the differences in multiple comparisons was determined using the one-way analysis of variance (ANOVA). Spearman's correlation analysis was used to assess the association of mRNA expression. $P < 0.05$ was considered statistically significant.

Results

LncRNA LEF1-AS1 and LEF1 were highly expressed in CRC tissues and cell lines

To determine the expressional pattern of LEF1-AS1 and LEF1 in CRC, CRC tissues and adjacent tissues was analyzed using qRT-PCR. LEF1-AS1 and LEF1 expression were higher in CRC tissues than that in adjacent tissues (Fig. 1a-b). By using bioinformatics database (<http://www.ncbi.nlm.nih.gov>), locations of LEF1 and LEF1-AS1 in the genome are shown as in Fig.1c. LEF1-AS1 was transcribed from the promoter region of LEF1, with a conversed orientation of LEF1. There was a positive correlation between the expression of LEF1-AS1 and LEF1 in clinical CRC tissues (Fig. 1d). The correlation between LEF1-AS1 and LEF1 was further validated by qRT-PCR in several types of cells. Consistent with the clinical results, upregulated LEF1-AS1 and LEF1 were determined in high metastatic cell lines SW620 and LOVO (Fig.1e). LEF1-AS1 transcript was observed in the nucleus of SW620 and SW480 cells lines through subcellular fractionation (Fig. 1f). The above results indicated that highly expressed LEF1-AS1 and LEF1 might be related to the progression of CRC.

LEF1-AS1 mediate LEF1 expression by epigenetic modification

To investigate the correlation between LEF1-AS1 and LEF1 expression, rescue assays were conducted in LEF1-AS1/LEF1-silenced SW620 cells and LEF1-AS1/LEF1-overexpressed SW480 cells. Firstly, we found that the levels of LEF1-AS1 and LEF1 were noticeably increased in LEF1-AS1-silenced SW620 cells with LEF1-AS1 co-transfection. LEF1 level was significantly enhanced in LEF1-AS1-silenced SW620 cells with LEF1 co-transfection, while no change in LEF1-AS1 level. LEF1-AS1 and LEF1 levels showed the same tendency in LEF1-silenced SW620 cells with LEF1-AS1 or LEF1 co-transfection (Fig.2a). LEF1 protein levels were also analyzed in LEF1-AS1/LEF1-silenced SW620 cells with LEF1-AS1 or LEF1 co-transfection (Fig.2b). On the contrary, downregulation of LEF1-AS1 significantly restrained both LEF1-AS1 and LEF1 expression in LEF1-AS1- or LEF1-overexpressed SW480 cells. More importantly, inhibition of LEF1 led to an overt confinement in LEF1 level but not LEF1-AS1 in LEF1-AS1- or LEF1-overexpressed SW480 cells (Fig.2c). LEF1 protein levels were also analyzed in LEF1-AS1/LEF1-overexpressed SW480 cells with siLEF1-AS1 or siLEF1 co-transfection (Fig.2d). These results suggested that the expression of LEF1 was regulated by LEF1-AS1 in CRC cell lines.

We next assessed the specific mechanisms that LEF1-AS1 mediated LEF1. To test this, we determined the presence of mark at the LEF1 promoter, dimethylation at Lys4 of histone 3 (H3K4me3), which was associated with promoter activation[22]. MLL1, a major methyltransferase responsible for H3K4 trimethylation, modified H3K4me3 around the transcription start site of LEF1. We hypothesized that repression of LEF1 transcription by siLEF1-AS1 was also associated with a switch in the histone modification marks at the LEF1 promoter. LEF1-AS1 interacted with MLL1 was predicted by bioinformatics analysis (<http://pridb.gdcb.iastate.edu/>). As seen in Fig. 2e, the LEF1 promoter was present to a much lower extent in siLEF1-AS1 cells than in vector control cells by ChIP analysis using anti-H3K4me3 antibody. MLL1 silence inhibited LEF1 mRNA level in both SW480 and SW620 cells (Fig.2f). The same results were obtained for LEF1 promoter, which was shown in siMLL1 cells with anti-MLL1 or anti-H3K4me3 antibodies (Fig.2g). RIP analysis further confirmed that LEF1-AS1 RNA could be pulled down by MLL1 antibody in both SW480 and SW620 cells (Fig.2h). The levels of LEF1 mRNA and protein were decreased after MLL1 or LEF1-AS1 knockdown (Fig.2i). These results suggested that LEF1-AS1 was a mediator in the H3K4me3 methylating process of LEF1 promoter region.

LEF1 regulates FUT8 expression in CRC cells

In our previous research, FUT4, FUT5 and FUT6 were found to promote the development of CRC. We wondered if other genes in FUT family that play an important role in CRC progression. First, the expressional profile of FUT genes was analyzed by qRT-PCR in CRC tissues. Higher level of FUT8 was measured in CRC tissues than that in adjacent tissues (Fig. 3a). The expression of FUT8 protein in CRC tissues showed the same tendency (Fig. 3b). Furthermore, the positive correlation between the expression of FUT8 and LEF1 in CRC tissues was shown in Fig. 3c. We further investigate FUT8 levels by manipulating LEF1 expression in CRC cells. As shown in Fig. 3E, force expression of LEF1 enhanced FUT8 level, whereas of LEF1 knockdown remarkably reduced FUT8 expression (Fig.3d). In the adjacent tissues and SW480 cells with a lower expression of LEF1, FUT8 level was obviously lower than that in CRC tissues and SW620 cells with higher expressed LEF1 (Fig.3e).

In Fig.3f, the genomic structure of human FUT8 gene was shown. FUT8 contains 11 exons and the translation initiation site is located on exon 3. The promoter upstream of exon 1 had the highest promoter activity[23]. Computer promoter analysis of the FUT8 5'-UTR revealed that the region of promoter 1 contained multiple LEF1 binding sites. To further determine whether the LEF1 protein directly binds to the FUT8 promoter, EMSA was performed. The biotin-labeled wild-type FUT8 probe oligonucleotides bound to the purified LEF1 protein extracted from SW620 cells (Fig. 3g). To further ultimately demonstrate that LEF1 actually binds to the FUT8, ChIP assay was carried out. Chromatin from SW480 and SW620 cells were immunoprecipitated with rabbit anti-LEF1 antibody or rabbit IgG, and the resulting immunoprecipitates were analyzed by PCR using specific FUT8 primers. As shown in Fig. 3h, an obvious band, the amplified fragment of the FUT8, was observed in the anti-LEF1-IP sample compared to the IgG control. Therefore, LEF1 and FUT8 levels were showed closely correlation.

FUT8 promotes the core fucosylation of CRC cells via LEF1-AS1/LEF1- activated Wnt/ β -catenin pathway

Emerging evidence shows that core fucosylation plays an important role in regulating protein activity involved in tumor growth and tumor dissemination[24, 25]. Here, lens culinaris agglutinin (LCA) was used to measured core fucosylation levels via lectin blot. As shown in Fig. 4a, overexpression of FUT8 enhanced, while knockdown of FUT8 inhibited the biosynthesis of core-fucosylated glycans in SW480 and SW620 cells. The core fucosylation level was altered on the transfected CRC cell surface via measuring FITC-LCA intensity by flow cytometry (Fig. 4b). The lectin histochemical staining with LCA in typical CRC tissues and adjacent tissues were performed. The expression of LCA-binding α 1, 6-fucosylated glycoproteins in CRC tissues was higher than that in adjacent tissues (Fig. 4cC).

It has been confirmed that FUT8 was up-regulated in non-small cell lung cancer via the transactivation of β -catenin/LEF-1[23]. Accordingly, we hypothesized that the active Wnt/ β -catenin pathway could promote FUT8 expression. Lithium chloride (LiCl) activates the β -catenin-mediated Wnt signaling pathway. The results showed that LiCl treatment led to increased protein and mRNA levels of FUT8 in time and dose-dependent manner in CRC cell lines (Fig. 4d).

To further determine whether LEF1-AS1/LEF1 could activate Wnt/ β -catenin pathway and participates in the regulation of FUT8 expression, GSK-3 β , (Ser9) p-GSK-3 β and β -catenin levels were measured by western blot in CRC cell lines. Compared with mock control, the (Ser9) p-GSK-3 β and β -catenin protein levels were significantly up-regulated by overexpression of LEF1-AS1. While knockdown of LEF1-AS1 dramatically decreased (Ser9) p-GSK-3 β and β -catenin protein levels (Fig. 4e). To measure nuclear and cytoplasmic β -catenin levels in CRC cells upon altered LEF1-AS1 expression, immunofluorescence assays were performed. The results indicated that LEF1-AS1 increased β -catenin nuclear accumulation in CRC cells (Fig. 4f). Normally, once β -catenin translocated into the nucleus, it could activate the expression of downstream target genes. Therefore, we conducted the TOP/FOP Flash luciferase reporter gene assay and found that LEF1-AS1 regulated the activities of β -catenin pathway activity in the CRC cell lines (Fig.4g). Subsequently, the molecular expression of downstream in Wnt/ β -catenin pathway was analyzed. LEF1-AS1 increased the protein levels of c-myc, cyclin D1, MMP-2, and MMP-9 (Fig. 4h). These

findings indicated that FUT8 significantly promoted core fucosylation in CRC cell lines through LEF1-AS1/LEF1 activating Wnt/ β -catenin signaling pathway.

LEF1-AS1 mediates the malignancy of CRC cell lines *in vitro*

Based on previous discoveries, we aspired to scrutinize the biological role of LEF1-As1 in CRC progression. LEF1-AS1 expression was upregulated in SW480 and HCT-8 cell lines expressing low endogenous LEF1-AS1, while LEF1-AS1 targeting siRNA and corresponding controls (siSCR) were used to knock down LEF1-AS1 in SW620 and LOVO cells with high endogenous LEF1-AS1 expression. Then the effects of LEF1-AS1 on the CRC proliferation were assessed using the CCK-8, colony formation and EDU assays. The overexpression of LEF1-AS1 in SW480 and HCT-8 cells caused a crucial increase in cell proliferation (Fig. 5a-c). Furthermore, LEF1-AS1 overexpression in CRC cells resulted in enhanced cell migration and invasion (Fig. 5d). Conversely, LEF1-AS1 knockdown in SW620 and LOVO cells inhibited the cell proliferation compared with siSCR control (Fig. 5e-g). LEF1-AS1 silence attenuated migration and invasion in CRC cells estimated by wound healing and transwell assays (Fig. 5h), suggesting that LEF1-AS1 was a positive regulator of CRC progression.

LEF1-AS1/LEF1/FUT8 mediates CRC malignancy *in vivo*

To assess the potential function of LEF1-AS1/LEF1/FUT8 on tumor growth *in vivo*, different treatment SW620 cells were injected subcutaneously into the nude mice. The nude mice were divided into four groups. In vivo tumorigenicity experiments, compared with control group, the tumor growth was significantly inhibited by shLEF1-AS1, while promoted by FUT8 overexpression. Co-transfection of shLEF1-AS1 and FUT8 showed a markedly increase in tumor growth compared to the shLEF1-AS1 group, suggesting that FUT8 overexpression partially reversed the inhibitory effect of shLEF1-AS1 on SW620 cell tumorigenicity (Fig. 6a-c). Then we evaluated the FUT8 mRNA and protein levels in tumors. Ki67 expression in different groups was also assessed by immunohistochemistry (Fig. 6d-e).

To further examine the influence of LEF1-AS1 and FUT8 on tumor metastasis *in vivo*, different treatment SW620 cells were injected into nude mice via the spleen and tail vein. The shLEF1-AS1 group showed decreased liver and lung metastasis compared with the control group, while FUT8 group showed the opposite tendency. Compared with shLEF1-AS1 group, the liver and lung metastasis of SW620 cells were advanced in treatment of co-transfection of shLEF1-AS1 and FUT8 (Fig. 6f-i). These findings provided promising therapeutic targets for CRC development.

Discussion

Tumor metastasis remains the leading cause of treatment failure and death in CRC patients. The discovery of new biomarkers is critical for predicting disease progression at an early stage. Herein, our current work was conducted to verify the oncogenic potential of LEF1-AS1/LEF1/FUT8 in association with fucosylation and Wnt/ β -catenin pathway in CRC progression.

In recent years the function of lncRNAs has started to be unveiled in CRC. Human transcriptome sequencing identifies a great number of dysregulated lncRNAs in CRC, some of which play essential roles in tumorigenesis and progression[26]. Among them, lncRNA LEF1-AS1 is reported to be upregulated and correlated with lymph node metastasis and poor survival in colon cancer patients[6, 27]. The function of LEF1-AS1 in pancreatic and colorectal carcinoma cell lines is dependent on the unspliced transcript and ultimately on the regulation of LEF1[28]. LEF1-AS1 is an antisense lncRNA derived from the promoter region of LEF1. Our current study showed that LEF1-AS1 and LEF1 were markedly higher in CRC tissues and high metastatic CRC cells than that in adjacent tissues and normal cells. Intriguingly, LEF1-AS1 was found to be positively correlated with LEF1 in CRC. LEF1-AS1 also modulated LEF1 expression. Unexpectedly, we could not detect any significant alteration of LEF1-AS1 expression after altered levels of LEF1. LEF1-AS1 regulated H3K4 trimethylation in LEF1 promoter region by binding to and recruiting MLL1. H3K4me3 transition in LEF1 promoter could alter the chromatin status from a closed and inactive state to an open and active state, and stimulate LEF1 transcription. These findings indicated that lncRNA LEF1-AS1 might serve as a good potential prognosis maker for metastatic CRC patients in transcriptional level.

The FUT family is frequently found to be deregulated in diverse types of cancers, resulting in a high mortality rate[25, 29, 30]. Certain reports have shown that FUT8 promotes cell metastasis in Melanoma and nonsmall cell lung cancer[14, 23]. In our previous study, we found that FUT8 expression was significantly up-regulate in high metastatic hepatocellular carcinoma cells[16]. To further investigate the effect of FUT8 in CRC, we measured the FUT8 expression level in CRC tissues and cells relative to non-tumor tissues and normal cells. Consistent with the previous findings, a significant increase in FUT8 was observed in CRC tumor tissues and cells. Chen et al. showed that the nuclear β -catenin cooperated with LEF-1 to activate FUT8 expression, which promoted the progression of non-small cell lung cancer[23]. Here, the positive correlation between the expression of FUT8 and LEF1 in CRC tissues was shown. LEF1 also regulated FUT8 expression.

Aberrant glycosylation is considered to be a hallmark of cancer. Accumulating evidence suggests that the core fucosylation plays an important role in cancer. Core fucosylation is critical for the function of TGF- β receptor by affecting the binding affinity toward TGF- β [31]. The enhanced core fucosylation by FUT8 promotes epithelial-mesenchymal transition in non-small cell lung cancer cells[23]. Yang et al reveal that up-regulation of FUT8 and α 1, 6-Fcosylation in breast cancer promotes cell stemness and EMT via Wnt/ β -catenin signaling pathway[15]. Aberrant activation of Wnt/ β -catenin signaling pathway is associated with a variety of pathological alterations, including cancer[15]. In the current study, FUT8 promoted the core fucosylation of glycoproteins in CRC cells. The expression of FUT8 was significantly enhanced by treatment with Wnt/ β -catenin signaling activator LiCl. He et al. showed that LEF1-AS1 mediated retinoblastoma progression through regulating β -catenin and LEF1 expression[32]. Our results provided evidence that LEF1-AS1 mediated FUT8 level through activation of Wnt/ β -catenin/LEF1 pathway, thereby resulting in β -catenin nuclear translocation. LEF1-AS1 also increased the protein levels of c-myc, cyclin D1, MMP-7 in CRC cells. These data suggest that LEF1-AS1/LEF1 mediated the upregulated FUT8 and α 1,

6-fucosylation level via Wnt/ β -catenin pathway, and FUT8 and α 1,6-fucosylation might serve as potential targets for CRC development.

Accumulated evidences demonstrate that LEF1-AS1 is enrolled in the tumorigenesis and progression of a variety of cancers. Furthermore, several recent studies have indicated that LEF1-AS1 is upregulated human CRC tissues and cell lines, and correlated with the overall and recurrent-free survival of colon cancer patients[27, 33, 34]. These results indicate that LEF1-AS1 may act like oncogene in cancers. In our study, we found that enforced LEF1-AS1 expression in CRC cells promoted proliferation, migration, and invasion, as well as LEF1-AS1 knockdown suppressed the progression of CRC cells *in vitro*, suggesting that LEF1-AS1 behaved like oncogene in CRC, too. Additionally, exogenous introduction of FUT8 could elevate the effects of LEF1-AS1 silence on tumor xenograft growth, liver and lung metastasis. Our results provided strengthen evidence that FUT8 acted as a driver in CRC progression mediated by LEF1-AS1/LEF1/FUT8 axis via Wnt/ β -catenin pathway.

Conclusions

In summary, we reported a regulatory network for core fucosylation in CRC. The identification of this regulatory network enhanced our knowledge to elucidate the molecular mechanism of CRC development. Targeting LEF1-AS1/LEF1/FUT8 axis might improve the outcomes of therapy in CRC patients. This might provide a potential therapeutic strategy for treating CRC.

Abbreviations

CRC: colorectal cancer ;LncRNAs: Long non-coding RNAs; miRNAs: MicroRNAs; LEF1-AS1: Lymphoid enhancer-binding factor 1 antisense RNA 1; GTs: glycosyltransferases; FUTs: fucosyltransferases

Declarations

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Authors' contributions

YQ and YS are responsible for conducting experiments, acquisition of data, analysis and drafted the manuscript. SL and YH provided technical and material support. YG and TH collected the clinical data. LJ was responsible for designing the experiments and research supervision. All authors read and approve the final manuscript.

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Availability of data and materials

Our data, related material and experimental methods were available from the authors based on reasonable request.

Ethics approval and consent to participate

The study has been examined and certified by the Ethics Committee of the First Affiliated Hospital of Dalian Medical University (Ethics Reference NO: YJKY-FB-2016-16), and informed consent was obtained from all participants included in the study, in agreement with institutional guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

LncRNA LEF1-AS1 and LEF1 were highly expressed in CRC tissues and cell lines. (a, b) LEF1-AS1 and LEF1 levels were analyzed in CRC tissues and their corresponding adjacent tissues by qRT-PCR. (c) Schematic diagram of LEF1-AS1 and LEF1 structures was shown. LEF1-AS1 was transcribed from the promoter region of LEF1-AS1, with a conversed orientation of LEF1. (d) A positive correlation was observed between the expression of LEF1-AS1 and LEF1 in clinical CRC tissues. (e) The expression of LEF1-AS1 and LEF1 was analyzed in FHC, HCT-116, CaCo2, SW480, SW620, LOVO cell lines by qRT-PCR. (f) Cellular localization of LEF1-AS1 in CRC cells was shown. GAPDH and U6 served as a cytoplasmic and nuclear localization marker, respectively.

Figure 2

LEF1-AS1 repressed LEF1 expression by epigenetic modification. (a, b) In SW620-siLEF1-AS1 or SW620-siLEF1 cells, LEF1-AS1 overexpression increased LEF1 level while overexpressed LEF1 had no effect on LEF1-AS1 level by qRT-PCR and western blot. (c, d) In SW480-LEF1-AS1 or SW480-LEF1 cells, knockdown of LEF1-AS1 inhibited LEF1 expression while LEF1 silence had no effect on LEF1-AS1 level by qRT-PCR and western blot. (e) Knockdown of LEF1-AS1 decreased H3K4me3 modification in the region of LEF1 promoter by ChIP analysis. (f) MLL1 mediated the transcription of LEF1 by combining with its promoter. RT-PCR showed that siMLL1 decreased LEF1 mRNA level in both SW480 and SW620 cells. (g) Knockdown of MLL1 attenuated LEF1-pro by ChIP analysis. (h) The combination of LEF1-AS1 and MLL1 was shown by RIP assays. (i) The levels of LEF1 mRNA and protein were decreased after MLL1 knockdown or LEF1-AS1 knockdown. The error bars in all graphs represented SD, and each experiment was repeated three times. * $p < 0.05$

Figure 3

LEF1 regulated FUT8 expression in CRC. (a) The mRNA levels of FUT family were analyzed by qRT-PCR in 36 pairs of CRC tissues and their corresponding adjacent tissues. The relative amount of mRNA level was normalized to the adjacent tissue. (b) FUT8 protein levels were increased in CRC tissues by western blot analysis. (c) A positive correlation was observed between the levels of FUT8 and LEF1. (d) Altered LEF1 regulated the protein levels of FUT8 in CRC cell lines. (e) In adjacent tissues or SW480 cells with a low expression of LEF1, FUT8 level was obviously lower than that in CRC tissues or SW620 cells with highly expressed LEF1. (f) Genomic structure of human FUT8 gene. FUT8 contains 11 exons and the translation start site was located on exon 3. (g) The binding of LEF1 to FUT8 promoter was demonstrated by EMSA assay. (h) ChIP assay with an anti-LEF1 antibody followed by the PCR detection of ChIP DNA with FUT8 promoter primers. IgG was used as a negative control, chromatin extracts were used as input. Error bars represent the mean $\pm$ S.D. of triplicate experiments. $*P < 0.05$.

Figure 4

FUT8 promoted the core fucosylation of CRC cells via LEF1-AS1/LEF1-activated Wnt/β-catenin pathway. (a) The transfected CRC cells were analyzed by SDS-PAGE and stained with Coomassie brilliant blue (CBB; left panel), and LCA lectin blot (right panel). (b) The transfected CRC cells were stained with FITC-conjugated LCA and analyzed with flow cytometry. (c) Lectin-histochemical staining with LCA in adjacent tissues and CRC tissues. (d) FUT8 expression was elevated in a time- and dose-dependent manner upon

LiCl treatment at both protein and mRNA levels. (e) The effect of LEF-AS1 on Wnt/ β -catenin pathway, LEF1 and FUT8 was detected by western blot. (f) Subcellular localization of β -catenin in both LEF1-AS1-SW480 and siLEF1-AS1-SW620 cells were analyzed. β -catenin was stained as red in both subcellular and nuclei compartment. The nuclei were stained by DAPI as blue in the figure. (g) Luciferase activity of TOP and FOP in CRC transfected cells was detected. (h) c-myc, cyclin D1, MMP-2 and MMP-9 levels after transfection were assessed by western blot with corresponding antibodies. Error bars represent the mean $\pm$ S.D. of triplicate experiments. * $P < 0.05$.

Figure 5

Manipulation of LEF1-AS1 expression regulated CRC proliferation, migration and invasion in vitro. (a, d, c) The effect of LEF1-AS1 knockdown on proliferation of SW620 and LOVO cells were determined by CCK-8 assay, colony formation assay and immunofluorescence analysis with EDU. (d) Downregulation of LEF1-AS1 inhibited SW620 and LOVO cells migration and invasion based on wound healing assay and transwell assay. (e, f, g) The effect of LEF1-AS1 overexpression on proliferation of SW480 and HCT-8 cells were determined by CCK-8 assay, colony formation assay and immunofluorescence analysis with EDU. (h) Overexpression of LEF1-AS1 promoted SW480 and HCT-8 cells migration and invasion based on wound healing assay and transwell assay. Error bars represent the mean $\pm$ S.D. of triplicate experiments. * $P < 0.05$.

Figure 6

LEF1-AS1/LEF1/FUT8 axis regulated the growth and metastasis of CRC in vivo. (a) Images of the tumor lumps in each group at the endpoint of the experiment. (b) Tumor volume curve of each group were analyzed ($n = 5$, $*P < 0.05$). (c) Tumor weights of each group were measured. (d) The levels of FUT8 in tumor tissues were assessed by qRT-PCR. (e) Immunohistochemical staining of FUT8 and Ki-67 levels was determined. (f) Representative images of GFP signals of mice in each group after spleen and tail vein injection with 2×10^6 SW620 cells in each group. (g) The liver, spleen and lung sections were shown via HE staining. (h) The number of metastasis in the liver and lung were determined. (i) FUT8 expression was examined in liver and lung metastasis tissues by qRT-PCR. The error bars in all graphs represented SD. $*p < 0.05$.