Long noncoding RNA BFAL1 mediates enterotoxigenic Bacteroides fragilis-related carcinogenesis in colorectal cancer via the RHEB/mTOR pathway

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Abstract
Long noncoding RNAs (lncRNAs) contribute to many steps in carcinogenesis and often serve as biomarkers or therapeutic targets for tumor diagnosis and therapy. Although the role of lncRNAs in tumor formation is becoming clear, whether lncRNAs mediate gut microbiota-induced colorectal cancer (CRC) is largely unknown. Enterotoxigenic Bacteroides fragilis (ETBF) is a well-known tumor-inducing bacterium in the human gut; however, its tumorigenic effect remains to be explored. In the present study, we revealed the mechanism by which a lncRNA participates in gut bacteria-induced carcinogenesis: Bacteroides fragilis-associated lncRNA1 (BFAL1) in CRC tissues mediates ETBF carcinogenesis. BFAL1 was highly expressed in CRC tissues compared with that in adjacent normal tissues. In vitro, BFAL1 was upregulated in ETBF-treated CRC cells. Mechanistically, ETBF promoted tumor growth via BFAL1 by activating the Ras homolog, which is the MTORC1 binding/mammalian target of the rapamycin (RHEB/mTOR) pathway. Furthermore, BFAL1 regulated RHEB expression by competitively sponging microRNAs miR-155-5p and miR-200a-3p. Clinically, both high expression of BFAL1 and high abundance of ETBF in CRC tissues predicted poor outcomes for patients with CRC. Thus, BFAL1 is a mediator of ETBF-induced carcinogenesis and may be a potential therapeutic target for ETBF-induced CRC.

Introduction
CRC is one of the most common malignant tumors worldwide, occurring in 5% of the adult population in the United States. Around 250,000 new cases are diagnosed each year, accounting for ~9% of all malignancies in Europe1–4. CRC carcinogenesis is controlled by both genetic and environmental factors, in which the gut microbiota plays an important role in CRC formation5. Enterotoxigenic Bacteroides fragilis (ETBF) is one of the most prevalent species of carcinogenic bacteria in the colon6. ETBF is a subtype strain of Bacteroides fragilis, which possesses a bft gene, encoding Bacteroides Fragilis Toxin (BFT); the nontoxicogenic Bacteroides fragilis (NTBF) subtype lacks the toxin gene7,8. Previous studies revealed that BFT targets the epithelial cell tight junctions, resulting in E-cadherin cleavage, enhanced barrier permeability, and Wnt/β-catenin and nuclear factor kappa B (NF-κB) signaling9. A recent study showed that BFT promoted the normal-polyp-cancer process10. These mechanisms involved genetic mutations in various genes, such as ICAM1
(intercellular adhesion molecule 1), AR (androgen receptor), JNK (JUN N-terminal kinase), MAPK (mitogen-activated protein kinase), and NF-κB11–13.

Less than 2% of the human genome comprises protein-coding genes and the vast majority of transcripts consist of noncoding RNAs, representing a shift in our understanding of genome regulation that has emerged recently14. It is now apparent that the majority of cellular transcripts do not encode proteins, and many of these transcripts are long noncoding RNAs (lncRNAs)15. LncRNAs are transcribed mainly by RNA polymerase II, and are 5′-capped and polyadenylated like most RNAs, yet this class of transcripts has limited coding potential16. LncRNAs are involved in numerous biological functions and pathological processes, including development, proliferation, apoptosis, survival, differentiation, and carcinogenesis17,18, contributing to gene regulation by different mechanisms19. Among the reported mechanisms, some lncRNAs act post-transcriptionally as regulators of splicing, mRNA decay, protein translation, protein stability, or as molecular decoys for microRNAs20.

As omics-based technologies have matured, increasing evidence points to the microbial generation of bioactive compounds that affect the transcriptional machinery of host cells21. Recent studies have provided insights into the crosstalk between the gut microbiota and the host epigenome, including DNA methylation, histone modification, and noncoding RNAs22,23. Commensal microbiota-regulated lncRNAs have been identified in mouse models. Compared with germ-free mice, those that were colonized with specific bacteria displayed a significantly different lncRNA profile, with most of the lncRNAs being transcribed from introns. These lncRNAs contributed to the responses of intestinal epithelial cells to the bacteria24. However, these data were solely based upon the bioinformatic data. Therefore, we decided to identify an ETBF-associated lncRNA and explore its molecular mechanism in human CRC carcinogenesis to provide a potential therapeutic target for ETBF-induced CRC.

In the present study, Bacteroides fragilis-associated lncRNA1 (BFAL1) was identified. The expression profile of BFAL1 was validated and its function in ETBF-related carcinogenesis was investigated. BFAL1 mediates ETBF-induced tumor growth by activating the Ras homolog, which is the MTORC1 binding/mammalian target of the rapamycin (RHEB/mTOR) pathway. Further study showed that BFAL1 competitively bound to miRNAs miR-155-5p and miR-200a-3p to upregulate RHEB expression. Clinicopathological information from 96 patients with CRC suggested that BFAL1 was an independent indicator of prognosis. Thus, the present study might identify a new field of research into how noncoding RNAs respond to microbial signaling and promote CRC carcinogenesis.

**Materials and methods**

**CRC tissue specimens**

The use of human tissues was performed in accordance with the Declaration of Helsinki and was approved by the ethics committee of Renji Hospital. Written informed consent was obtained from all participants in this study. Cohort 1 represented adult patients with CRC who underwent primary colorectal surgical resections at Renji Hospital and were enrolled from January 2010 to April 2014. All patients were diagnosed as colorectal adenocarcinomas. None of these patients had received radiotherapy or chemotherapy before surgery. Paired tissues (tumors and adjacent normal tissues) were collected and preserved in liquid nitrogen immediately for subsequent study.

**Detection of the amounts of ETBF in paired CRC tissues**

To detect the amounts of ETBF in CRC tissues, the total DNA was extracted from the paired CRC tissues by using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). DNA from each specimen was subjected to quantitative real-time PCR (qPCR) to detect the amounts of ETBF. The detected amount of the bft gene was normalized to that of the 16S gene (Supplementary Material Table 1).

**Quantification of mRNAs and microRNAs**

The total RNA was isolated from cells by using the TRIzol reagent (Takara, Dalian, China) according to the manufacturer’s protocol. Separation of the nuclear and cytoplasmic fractions was performed by using a PARIS™ Kit (Invitrogen, Carlsbad, CA, USA). To obtain cDNA, 1 μg of RNA was used as a template, and reverse transcription was performed by using a PrimeScript 1st strand cDNA Synthesis Kit (Takara) according to the manufacturer’s instructions. Primers for LncRNAs and genes were designed and synthesized by Sangong Biotech, Shanghai, China (Supplementary Material Table 1). For miRNAs, 0.5 μg of the total RNA was reverse transcribed into cDNA by using a specific miRNA stem loop primer. The levels of mRNA and miRNA were assessed quantitatively by using SYBR Green-based qPCR with specifically designed primers (GeneCopoeia, Rockville, MD, USA) (Supplementary Material Table 2). All qPCR reactions were performed by using a 7500 Fast Real-Time PCR System (Applied Biosystems), and all qPCR reagents were purchased from Takara. For each reaction, 1 μL of the RT product was added to 10 μL of 2 × SYBGreen PCR Master Mix. Each sample was analyzed in triplicate. For lncRNAs and mRNAs, ACTB (encoding beta actin) was used as an internal normalization control, and for the miRNAs, U6 was used as the internal normalization control. Relative quantification (RQ) was derived from the difference in the cycle threshold (Ct) between the target
RNA and internal controls (ΔΔCt) as compared with control samples (ΔΔCt) by using the equation RQ = 2 – ΔΔCt.

Cell lines and cell culture

Human CRC cell lines and the human normal colonic epithelial cell line FHC were purchased from American Type Culture Collection (ATCC, Rockeferrer, MD, USA). Cells were cultured in a different medium according to the ATCC’s indication (Gibco, Carlsbad, CA, USA). Cells were cultured in 5% CO₂ in a 37 °C incubator.

Bacteria strains and the growth condition

The ETBF strain (ATCC 43860) and the NTBF strain ATCC 25285 were purchased from ATCC. These two subtypes were cultured in the same medium and under anaerobic conditions. The anaerobic conditions were created by using a DG250 device (Don Whiteley Scientific, West Yorkshire, UK) and comprised an atmosphere of 90% N₂, 5% CO₂, and 5% H₂ at 37 °C. The anaerobic bacteria medium was prepared according to the ATCC indication (modified chopped meat medium). All the ETBF and NTBF treatment experiments in this article used the same bacterial concentration: a multiplicity of infection (MOI) of 500.

Overexpression of BFAL1 in CRC cells

For ectopic expression, the full-length BFAL1 cDNA was subcloned into the vector pCDNA3.1 and transfected into HCT116 and DLD-1 cell lines by using the FuGENE HD transfection Reagent (Promega, Madison, WI, USA). The vectors were designed and constructed by Gene Pharma Company (Shanghai, China).

Transfection of small-interfering RNAs and microRNA mimics and inhibitors

The specified BFAL1 small-interfering RNAs (siRNAs) siRNA1 and siRNA2, and the specified RHEB small RNA, control siRNA were designed to knockdown BFAL1 or RHEB expression in cells. MiR-155-5p mimics and inhibitors and miR-200a-3p mimics and inhibitors were transfected to overexpress or knockdown the relevant microRNAs in cells. All these siRNA and miRNA mimics and inhibitors were designed and synthesized by Gene Pharma Company (Supplementary Material Table 3). Transfection was performed by using the DharmaFECT transfection reagent (GE, Boston, MA, USA) according to the manufacturer’s instructions.

Cell proliferation assays and the cell cycle test

To assay cell proliferation, the CCK-8 (Cell Counting Kit-8, Dojindo, Japan) assay was used according to the manufacturer’s instructions. Flow-cytometry analysis was conducted by using a BD LSR Fortessa instrument and PI/RNase Staining Buffer (BD Biosciences, Lake Franklin, NJ, USA) according to the manufacturer’s instructions. The percentages of cells in different phases of the cell cycle were analyzed by using Flowjo software.

Bioinformatic methods

The potential microRNAs targeting RHEB were predicted and validated by using several online databases with different bioinformatic algorithms, such as TargetScan and RNAhybrid. We predicted the potential target genes of miR-155-5p and miR-200a-3p mainly based on a combination of 3’ UTR binding sites of target genes and the seed regions of miR-155-5p and miR-200a-3p. Furthermore, the minimum free energy (MFE) values of miRNA–lncRNA hybridization were calculated by using RNAhybrid software to evaluate the binding potential between BFAL1 and miR-155-5p or miR-200a-3p.

High-throughput sequencing

For RNA sequencing (RNA-seq), libraries were generated from the total RNA by using TruSeq RNA Sample Preparation v2, according to the manufacturer’s protocol. Samples were sequenced on the Illumina HiScanSQ platform (Illumina, San Diego, CA, USA). Reads were mapped to the human genome (Hg19) by using TopHat v2.0.6 (Johns-Hopkins University, Baltimore, MD, USA), and mRNA quantification was performed by using Cuffdiff v2.0.2 (University of Maryland, College Park, MD, USA).

Luciferase reporter assay

To explore BFAL1’s effect on the RHEB promoter transcriptional activity, a pGL3-RHEB reporter plasmid was designed and constructed (GENEray Company, Shanghai, China). The pGL3-RHEB plasmid and the relevant BFAL1 siRNAs or the pCDNA3.1 plasmid were transferred together into cells. To investigate the RHEB 3’ UTR activity, three plasmids were designed and synthesized (GENEray): pmirGLO-RHEB 3’ UTR wild-type (WT) plasmid, pmirGLO-RHEB 3’ UTR miR-155-5p mut plasmid, and pmirGLO-RHEB 3’ UTR miR-200a-3p mut plasmid. Luciferase activity was measured by using a FLUOSstar device (Omega Engineering, Deckenpfronn, Germany), with the Dual-Luciferase reporter assay system (Promega).

Western blotting and antibodies

Proteins were extracted from cells after different treatments and quantified by using a BCA Protein Assay Kit (Thermo Fisher Scientific, West Palm Beach, FL, USA). Proteins (40–60 μg) were electrophoresed through 10% SDS polyacrylamide gels and then electrophoretically transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The primary antibodies included those recognizing RHEB (Abcam,
Cambridge, UK and Cell Signaling Technology, Danvers, MA, USA), p70S6 Kinase (CST), and Phospho-p70S6 Kinase (CST); β-actin (CST) was used as an endogenous reference. All the secondary antibodies were labeled with horseradish peroxidase (HRP) (Aksomics, Shanghai, China). The signal was detected by using an ECL kit (Pierce Biotech, Rockford, IL, USA).

ShRNA and adenovirus construction

The shRNAs used in animal experiments and all adenoviruses were constructed and purchased from Obio Technology Company (Shanghai, China); Control shRNA (Y001, Obio), BFAL1-shRNA1 (Y2276, Obio), BFAL1-shRNA2 (Y9601, Obio), BFAL1-overexpressing adenovirus (H8855, Obio), miR-155-5p adenovirus (H9491, Obio), and miR-200a-3p adenovirus (H9492, Obio).

In vivo experiments

All animal experiments were performed according to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals. Our study was approved by the Animal Care and Use Committee of Renji Hospital, School of Medicine, Shanghai Jiao Tong University. Five-week-old male BALB/c nude mice were obtained from the Experimental Animal Center of Shanghai Institute for Biological Sciences (Shanghai, China). Each mouse was injected with 5.0 × 10⁶ HCT116 cells subcutaneously at the right axilla to establish CRC xenograft tumors. Six days after inoculation, these mice were divided randomly into different groups for different treatments. Different treatments were delivered paratumorally at multiple points every 3 days. The tumor length and width were measured by using calipers every 3 days. The tumor volume was calculated and recorded using the volume formula (long dimension × wide dimension^2/2). After 21 days, all mice were killed and the subcutaneous xenograft tumors were excised and weighted. Finally, all tumors were kept in formalin for a further marker of proliferation Ki-67 (Ki-67) staining. The tumor volume and weight were presented as mean ± SD (n = 5 or 6).

Statistical analysis

Data were analyzed using Student’s t-test for comparisons between groups to determine the statistical significance. The Pearson chi-squared test was used to analyze the associations between the patient’s clinicopathological characteristics and ETBF abundance or BFAL1 expression. Kaplan–Meier analysis and the log-rank test were performed to evaluate patient survival. A Cox proportional hazard model was performed to assess the prognostic value of ETBF and BFAL1. The difference between the growth rates was determined using analysis of variance (ANOVA) with repeated measures analysis of variances. All statistical tests were performed using SPSS 20.0 statistical software (IBM Corp., Armonk, NY, USA) or Graphpad Prism 5.0 (Graphpad Software Inc., San Diego, CA, USA). Each experiment was repeated at least three times. All data were presented as the mean ± SD and were calculated from three separate experiments. The results were considered statistically significant when the two-tailed P value was <0.05.

Results

BFAL1 is highly expressed in ETBF-related CRC tissues and cells

To identify a specific IncRNA associated with ETBF-related CRC, the top 20 significant CRC-related IncRNAs (tumors vs. normal, false discovery rate (FDR) < 0.01, fold change > 1.5) were targeted in the GEO database GSE20916. After that, these 20 IncRNAs were further filtered in another GEO database GSE31737 (tumors vs. normal, FDR < 0.01) and finally we got the eight IncRNAs (Fig. S1A). To search for IncRNAs associated with ETBF among these eight candidates, two colorectal cancer cell lines, DLD-1 and HCT116, were treated with ETBF for up to 30 h. Interestingly, significantly increased expressions of IncRNAs AK096729 (BFAL1), AK001058, and AK098081 were detected in the ETBF-treated HCT116 and DLD-1 cells after 24 h (Fig. 1a, Fig. S1B), compared with those in NTBF or simple medium-treated cells. This phenomenon indicated that ETBF increases the expression of certain IncRNAs in CRC cells.

To identify the expression profile of these three ETBF-associated IncRNAs in CRC tissues, 96 cases (Renji Cohort 1) were adopted to test both the ETBF abundance and IncRNA expression levels in the cancer tissues paired with noncancerous tissues. The data showed that cancer tissues presented significantly higher expression levels of AK096729 (P = 0.0123, Fig. 1b), AK001058 (P = 0.0418), and AK098081 (P = 0.0289) (Fig. S1C) and simultaneously had a higher abundance of ETBF (P = 0.0004, Fig. 1c) compared with those in normal tissues. Furthermore, the correlation between the ETBF abundance and the expression of these IncRNAs in cancer tissues was detected. The results showed that cancer tissues with a relatively high ETBF colonization presented a higher enrichment of AK096729 (P = 0.01, Fig. 1d), whereas AK001058 and AK098081 levels were not obviously related with ETBF abundance (Fig. S1D). Therefore, we focused on AK096729 for further study and named it as Bacteroides fragilis-associated IncRNA1 (BFAL1).

In order to identify whether BFAL1 is a novel lncRNA, the full-length cDNA of BFAL1 was isolated by using 3′ and 5′ rapid amplification of cDNA ends (RACE) and sequenced (Fig. S1E). BFAL1 is located on chromosome 19 and is mainly distributed in the cell cytoplasm (Fig. S1F). To determine its noncoding character, the BFAL1 sequence was analyzed by using an ab initio ncRNA
Fig. 1 (See legend on next page.)
transcriptome predictor, which showed that its non-coding probability was 95.22%, whereas its coding probability was 4.78% (Fig. S1G). Analysis of BFAL1 ORF sequences using a coding potential calculator failed to find any protein-coding potential (Fig. S1H). In addition, we calculated its coding potential using PhyloCSF. The PhyloCSF score of BFAL1 was −774, indicating that BFAL1 is unlikely to encode any protein product. Analysis using qPCR showed a higher expression of BFAL1 in human CRC cells than in normal colorectal epithelial cells (FHC) (Fig. S1I).

Both ETBF and BFAL1 are associated with patients’ clinicopathology and outcomes

To analyze the relationship between BFAL1 and the clinicopathological features of CRC, correlation regression analysis was used. Detailed information regarding Renji Cohort 1 is shown in Table S1. The data showed that the expression of BFAL1 was positively related with tumor size (P = 0.000), tumor invasion (P = 0.009), and lymph node involvement (P = 0.024) (Fig. 1e). Similarly, ETBF abundance also showed a positive relationship with tumor size (P = 0.001) and invasion (P = 0.009) (Fig. 1f). We further examined the outcomes of these 96 cases. Kaplan–Meier analysis revealed that high expression of BFAL1 in tumor tissues was associated with a reduced overall survival (P = 0.0025; hazard ratio (HR) 2.656; 95% confidence interval (CI) 1.409–5.007) (Fig. 1g). Patients with high ETBF abundance in their tumor tissues also exhibited a poor outcome (P = 0.007; HR 2.351; 95% CI 1.281–4.462) (Fig. 1h). Univariate regression analysis showed that both ETBF abundance (P = 0.002; HR 2.358; 95% CI 1.230–4.522) and BFAL1 expression (P = 0.004; HR 2.642; 95% CI 1.361–5.129) had the potential to predict CRC prognosis (Fig. S1I). Further multivariate regression analysis showed that BFAL1 expression (P = 0.024; HR 2.178; 95% CI 1.109–4.277) and ETBF abundance (P = 0.041; HR 2.041; 95% CI 1.029–4.049) were two independent factors for CRC aggressiveness, with significant hazard ratios for predicting outcomes (Fig. 1i, j).

Receiver-operating characteristic (ROC) analysis illustrated that the area under curve (AUC) of the TNM-stage-based model combined with BFAL1-based prediction (0.7674) was higher than that of the single TNM-stage model (0.6881), as was the combination of the TNM stage and ETBF prediction (0.8424) (Fig. 1k). This suggested that the combination of BFAL1 or ETBF and TNM stage was more accurate to predict CRC prognosis than the TNM stage alone.

**BFAL1 mediates ETBF’s promotion of tumor growth in vitro and in vivo**

To evaluate the biological function of BFAL1 in CRC, we knocked down BFAL1 using BFAL1-siRNA1 in CRC cells and extracted RNA for RNA-seq analysis. A total of 14,737 downregulated genes and 15,913 upregulated genes (≥2-fold) were detected after the knockdown of BFAL1 in CRC cells (raw data are accessible via the GEO number: GSE129950). Gene set enrichment analysis (GSEA) revealed that the genes related to cell proliferation were reduced (normalized enrichment score (NES) = 1.80, P < 0.01; Fig. S2A) and cell cycle-associated pathways were downregulated (NES = 2.80, P < 0.01; Fig. S2B) in BFAL1 knockdown cells. Meanwhile, we also treated DLD-1 cells with ETBF for 24 h and extracted the total human RNA for RNA-seq analysis. We identified 350 upregulated and 154 downregulated genes (≥2-fold and FDR<0.05; raw data accessible via the GEO number: GSE130152). Gene ontology (GO) enrichment analysis also revealed that ETBF treatment regulated a set of genes associated with cell proliferation and the cell cycle (Fig. S2C). These two RNA-seq analyses suggested that both BFAL1 and ETBF have an effect on CRC cell growth.

To validate the biological function of BFAL1 and ETBF in vitro, CCK-8 assays were performed in HCT116 and DLD-1 cells. The results showed that ETBF enhanced cell proliferation, whereas NTBF and single-medium treatment had no effect on proliferation (Fig. 2a). Meanwhile, we tested the function of BFAL1 by overexpression from a BFAL1 plasmid (Fig. S2D) or knockdown with BFAL1-siRNA1/2 (Fig. S2E). Overexpression of BFAL1 promoted...
Fig. 2 ETBF exerts a biological function on CRC cell growth via BFAL1 in vitro and in vivo. 

a CCK-8 assay of ETBF-treated HCT116 cells and DLD-1 cells compared with NTBF or single bacterial medium-treated cells (n = 6, ANOVA, ***P < 0.001). 
b CCK-8 assay of BFAL1 overexpression and control cells (n = 6, ANOVA, ***P < 0.001). 
c CCK-8 assay of BFAL1 knockdown in HCT116 cells and DLD-1 cells (n = 6, ANOVA, ***P < 0.001). 
d CCK-8 assays of ETBF-treated, BFAL1 knockdown HCT116 cells and DLD-1 cells (n = 6, ANOVA, ***P < 0.001). 
e Cell cycle analysis of ETBF-treated HCT116 cells and DLD-1 cells (mean ± SD of three independent experiments; ANOVA, *P < 0.05). 
f Cell cycle analysis of BFAL1 knockdown of HCT116 cells and DLD-1 cells (mean ± SD of three independent experiments; ANOVA, *P < 0.05). 
g Xenograft tumors in the nude mouse model under different treatments (n = 5). 
h Statistical analysis of tumor sizes (mean ± SD, n = 5, ANOVA, **P < 0.01). 
i Tumor weights of different mouse groups (mean ± SD, n = 5, ANOVA, *P < 0.05, **P < 0.01)
cell proliferation (Fig. 2b), whereas BFAL1 knockdown suppressed cell proliferation (Fig. 2c). Interestingly, ETBF-induced proliferation was significantly suppressed by BFAL1 knockdown (Fig. 2d), indicating that the ability of ETBF to promote cell proliferation might depend on BFAL1. Flow cytometry assays were performed to test the cell cycle process affected by ETBF and BFAL1. The results showed that cell growth was accelerated by ETBF compared with treatment with NTBF (Fig. 2e). In addition, knockdown of BFAL1 blocked the cell cycle (Fig. 2f).

In vivo, a xenograft tumor model was established in BALB/c nude mice. We observed that ETBF-treated tumors were larger and heavier than those of the control, whereas BFAL1-shRNA1/2 virus-treated tumors were smaller and lighter. However, ETBF treatment could not rescue the tumor growth inhibited by BFAL1-shRNA1/2 virus treatment (Fig. 2g–i, Fig. S2G–I). Ki-67 staining was consistent with these observations (Fig. S2F, J). From these in vitro and in vivo experiments, we concluded that ETBF exerts its effects on CRC tumor growth through BFAL1.

**BFAL1 mediates ETBF-induced tumor growth by activating the RHEB/mTOR pathway**

To further explore the mechanism of BFAL1 in ETBF-related CRC cell growth, we reviewed the two RNA-Seq data sets mentioned above. Both Hallmark and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis in GSEA showed that knockdown of BFAL1 affected mTOR-associated gene sets (Fig. 3a). KEGG analysis of ETBF-treated cell data also demonstrated that the mTOR-signaling pathway is the significantly functional pathway (Fig. 3b). Taken together, the results suggested that the mTOR signaling is the common downstream pathway of both ETBF and BFAL1. We then explored how ETBF and BFAL1 activate the mTOR pathway. According to the KEGG analysis, the mTOR-signaling pathway in GSEA comprised 12 associated genes affected by BFAL1 knockdown: VEGFB, EIF4EBP1, PGE, CAB39L, MAPK1, FIGF, ULK2, PIK3CD, AKT3, EIF4E, MLST8, and RHEB (Table S2). To screen out the ETBF and BFAL1-targeted gene, we treated DLD-1 cells with ETBF for 24 h and extracted the cell’s RNA for gene expression analysis using qPCR. Among the 12 genes, only the RHEB mRNA level was upregulated by ETBF (Fig. 3c); the others showed no significant response to ETBF (Fig. S3A). Therefore, RHEB became our research focus. RHEB can bind directly to the mTOR complex and regulate the mTOR-signaling pathway by phosphorylating the p70S6 Kinase (S6K). Therefore, we hypothesized that ETBF and BFAL1 might target RHEB to regulate the mTOR pathway. First, we validated the qPCR result from the ETBF-treated cells by showing that overexpression of BFAL1 upregulated the RHEB mRNA level (Fig. 3d), whereas knockdown of BFAL1 downregulated its level (Fig. 3e). We then confirmed these findings using western blotting. The results demonstrated that the expression levels of RHEB and Phospho-S6k, but not the total S6K, were increased in ETBF-treated cells compared with those in NTBF-treated cells (Fig. 3f). Similar results were obtained in BFAL1-overexpressing cells (Fig. 3g). Conversely, the expression levels of RHEB and Phospho-S6k were decreased in BFAL1-knockdown cells (Fig. 3h). Furthermore, ETBF could not upregulate RHEB and Phospho-S6K levels after knockdown of BFAL1 in DLD-1 cells (Fig. 3i). Also, BFAL1 was not likely to upregulate P-S6K expression after RHEB knockdown (Fig. 3j).

**BFAL1 regulates RHEB expression by binding to miR-155-5p and miR-200a-3p**

We next explored the mechanism of how BFAL1 regulates the RHEB expression. The RHEB mRNA level was regulated by BFAL1 expression; therefore, we synthesized a recombinant luciferase reporter containing the RHEB promoter region to test the promoter activity. However, BFAL1 had no effect on the promoter activity of RHEB (Fig. S3B), suggesting that BFAL1 might regulate RHEB mRNA expression via a post-transcriptional mechanism. Recently, it has been reported that miRNAs direct sequence-specific cleavage of the target mRNA and repress its translation, and many RNA transcripts block this activity by sponging miRNA, permitting target mRNA translation as competing endogenous RNAs (ceRNA).

The miRNAs that might target RHEB were predicted by the TargetScan prediction algorithm (http://www.targetscan.org) and then were validated in RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid) to calculate the complete hybridization around the seed match with the RHEB 3’ UTR. Finally, miR-155-5p and miR-200a-3p were screened out, targeting the RHEB 3’ UTR and also showed high MFE values of hybridization with BFAL1 (Fig. 4a). Several studies have reported that miR-155-5p and miR-200a-3p target RHEB mRNA.

In the present study, mimics and inhibitory forms of miR-155-5p and miR-200a-3p were synthesized and transfected into CRC cells. Inhibitors of miR-155-5p and miR-200a-3p increased both the mRNA (Fig. 4b) and protein levels (Fig. 4c) of RHEB, whereas their mimics decreased RHEB mRNA and protein levels (Fig. 4d, e). To further test the miRNA effect on the 3’ UTR of RHEB, pmirGLO-RHEB wild-type plasmids and inhibitors of miR-155-5p and miR-200a-3p were co-transfected into CRC cells. The results showed that the RHEB 3’ UTR activity was significantly enhanced by the miRNA inhibitors, not the mutated one (Fig. 4f–h). By contrast, mimics of miR-155-5p and miR-200a-3p exerted the opposite effect on RHEB 3’ UTR activity reporter (Fig. 4i and j). Thus, we
Fig. 3 ETBF activates the RHEB/mTOR-signaling pathway via BFAL1 in CRC. a GSEA analysis: enrichment hallmark of mTORC1 signaling (NES = 2.05, P = 0.00) and KEGG mTOR-signaling pathway (NES = 1.45, P < 0.05). b KEGG pathway analysis of ETBF-treated DLD-1 cells. (c) The mRNA level of RHEB in ETBF-treated HCT116 cells and DLD-1 cells compared with that in NTBF-treated cells. d The RHEB mRNA level in BFAL1-overexpressing HCT116 cells and DLD-1 cells. e The RHEB mRNA level in BFAL1-knockdown HCT116 cells and DLD-1 cells. f The protein expression of the RHEB/mTOR pathway in ETBF-treated HCT116 cells and DLD-1 cells, compared with that in NTBF-treated cells. g The expression of the RHEB/mTOR pathway in BFAL1-overexpressing HCT116 cells and DLD-1 cells. h The expression of the RHEB/mTOR pathway in BFAL1-knockdown HCT116 cells and DLD-1 cells. i The expression of the RHEB/mTOR pathway in DLD-1 cells treated with ETBF after BFAL1 knockdown. j The expression of the RHEB/mTOR pathway in HCT116 cells overexpressing BFAL1 after RHEB knockdown.
Fig. 4 (See legend on next page.)
confirmed that both miR-155-5p and miR-200a-3p target the 3’ UTR of RHEB to repress its expression.

Next, we investigated whether BFAL1 regulated the RHEB expression via miR-155-5p and miR-200a-3p. qPCR analysis showed that overexpression of BFAL1 decreased miR-155-5p and miR-200a-3p mRNA levels (Fig. 5a), whereas knockdown of BFAL1 increased their levels (Fig. 5b). Furthermore, luciferase reporter assays revealed that overexpression of BFAL1 led to activation of the RHEB 3’ UTR (Fig. 5c), whereas knockdown of BFAL1 led to deactivation of the RHEB 3’ UTR (Fig. 5d). Collectively, the results strongly suggested that BFAL1 sponged miR-155-5p and miR-200a-3p to regulate RHEB expression acting as a ceRNA.

In vivo, we also confirmed that miR-155-5p and miR-200a-3p had biological functions using a xenograft tumor model. The results showed that transfection with viruses expressing miR-155-5p and miR-200a-3p obviously attenuated tumor growth compared with the controls. Although BFAL1-overexpressing virus and ETBF treatment enhanced tumor growth, they could not entirely recover the growth retardation induced by the miR-155-5p and miR-200a-3p mimics (Fig. 5e–g, Fig. S3C). Collectively, these results confirmed that BFAL1 binds competitively with miR-155-5p and miR-200a-3p to attenuate their suppressive function on RHEB expression.

In conclusion, we highlighted a new signaling cascade of ETBF–BFAL1–RHEB/mTOR that promotes tumor growth in CRC (Fig. 6).

**Discussion**

The gut microbiota, as a complicated symbiotic organ in mammals, has marked effects on host health and disease. The gut microbiome provides environmental cues to which the host responds via alterations in the host epigenome and gene expression. Previous studies have focused on the roles of protein-coding genes. However, increasing evidence has demonstrated a crosstalk between the microbiota and epigenetic alterations. DNA methylation, histone modifications, and various noncoding RNAs are involved in bacteria-related cancers. However, the regulatory mechanism remains largely unknown. ETBF is regarded as one of the most prominent bacterial species in human CRC. Although it has been studied for many years, the mechanism by which ETBF alters the host’s noncoding RNAs remains poorly understood. Through a series of genomic, bioinformatic, biological, xenograft model, and clinical studies, we identified that IncRNA BFAL1 is upregulated by ETBF and revealed a new mechanism by which BFAL1 participates in ETBF-induced CRC formation.

A study from Turkey has examined ETBF in a CRC population. Using lbf as a marker, ETBF was detected more frequently in the stools of consecutive cases of CRC compared with that in concurrent hospital-based, age-, and gender-matched patients without CRC (38% ETBF in 73 cases of CRC and 12% ETBF in 59 controls, P < 0.01). In the present study, we demonstrated a higher abundance of ETBF in CRC tissues compared with that in paired-matched normal tissues. Furthermore, we revealed high expression of BFAL1 in cancer tissues, which correlated positively with ETBF abundance. These findings indicated that direct exposure of colorectal epithelial cells (CECs) to ETBF may result in the upregulation of certain IncRNAs, including BFAL1 in CECs. In CRC cells, we observed that the BFAL1 mRNA level was upregulated by ETBF treatment. Therefore, we hypothesized that BFAL1 is responsible for ETBF stimulation of CRC and focused on BFAL1 for further study.

In a clinical study, we found that ETBF and BFAL1 have similar clinicopathological effects on tumor size. In addition, patients with high levels of ETBF and BFAL1 expression had a poor prognosis. These findings strongly suggested the emerging prognostic and therapeutic value of ETBF and BFAL1.

Initiation and progression of CRC refers to events yielding biological changes that foster CEC proliferation and multiple gene mutations or epigenetic alterations, ultimately resulting in the transformation to cancer. However, the events that precipitate in this process remain unknown. The microbiome is a prime suspect for triggering the initiation and/or progression of CRC carcinogenesis. Recent studies have demonstrated that the gut microbiota exerts various biological functions on tumor formation and progression. *Fusobacterium nucleatum* was confirmed to induce autophagy and mediate...
Fig. 5 (See legend on next page.)
Unidentified metabolic and nutrients, and coordinates cell integrates intracellular and extracellular events concerning human malignancies. This pathway acts as a sensor that is reported to be dysregulated in ~50% of all CRC carcinogenesis. The mTOR-signaling pathway is mediated by the binding competitively to miR-155-5p and miR-200a-3p, potentially acting as a ceRNA. Recent studies have provided evidence that gut microbiota might modulate miRNAs to exert biological functions. For example, E. nucleatum might modulate the expression of miR-18* and miR-4082 and Escherichia coli might modulate miR-30C and miR-130A to regulate human colonic epithelial cell autophagy. By contrast, lncRNAs have been reported to bind with particular miRNAs to attenuate their suppressive effect on the target gene. For example, precise regulation by lncRNA uc.173 was demonstrated in the control of gut permeability by decreasing the availability of miR-29b to regulate CLDN1 mRNA expression. In the present study, we confirmed that BFAL1 regulates RHEB expression by binding to miR-155-5p and miR-200a-3p in ETBF-associated carcinogenesis.

Taken together, ETBF orchestrates the BFAL1, miR-155-5p/miR-200a-3p, and the RHEB/mTOR pathways to regulate CRC tumor growth. LncRNA BFAL1 might be a promising target for CRC diagnosis and therapy.

CRC chemoresistance, and is also associated with metastasis of primary CRC. The present study revealed that ETBF exerts a marked effect on CRC tumor growth in vitro and in vivo. However, when BFAL1 was knocked down, ETBF lost its biological effect on CRC, suggesting that BFAL1 mediates ETBF’s carcinogenic function in CRC.

Further mechanistic investigations revealed a hitherto unidentified signaling cascade of ETBF–BFAL1 RHEB/mTOR in CRC carcinogenesis. The mTOR-signaling pathway is reported to be dysregulated in ~50% of all human malignancies. This pathway acts as a sensor that integrates intracellular and extracellular events concerning metabolism and nutrients, and coordinates cell growth and autophagy. RHEB is a regulator of mTOR and is indispensable for mTOR activation in response to all stimuli. RHEB binds to mTOR on the lysosome surface and inactivates downstream signaling. mTOR signaling is rarely reported in microbiota-associated CRC issues. Our findings revealed the exact mechanism of the ETBF-induced mTOR pathway mediated by BFAL1. BFAL1 regulates RHEB expression by binding competitively to miR-155-5p and miR-200a-3p, potentially acting as a ceRNA. Recent studies have provided evidence that gut microbiota might modulate miRNAs to exert biological functions. For example, E. nucleatum might modulate the expression of miR-18* and miR-4082 and Escherichia coli might modulate miR-30C and miR-130A to regulate human colonic epithelial cell autophagy. By contrast, lncRNAs have been reported to bind with particular miRNAs to attenuate their suppressive effect on the target gene. For example, precise regulation by lncRNA uc.173 was demonstrated in the control of gut permeability by decreasing the availability of miR-29b to regulate CLDN1 mRNA expression. In the present study, we confirmed that BFAL1 regulates RHEB expression by binding to miR-155-5p and miR-200a-3p in ETBF-associated carcinogenesis.

Taken together, ETBF orchestrates the BFAL1, miR-155-5p/miR-200a-3p, and the RHEB/mTOR pathways to regulate CRC tumor growth. LncRNA BFAL1 might be a promising target for CRC diagnosis and therapy.

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Conflict of interest
The authors declare that they have no conflict of interest.

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