The hsa_circ_0000523/miR-let-7b/METLL3 Axis Regulates the Proliferation, Apoptosis and Metastasis of Human Colorectal Cancer Cell Line HCT116

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Research Article

Keywords: hsa_circ_0000523, METTL3, colorectal cancer, HCT116

Posted Date: January 20th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-142499/v1

Abstract

Background: Circular RNAs (circRNAs) have gained wide attention as a class of potential biomarkers for the early detection of multiple cancers. However, the functions and mechanisms of circRNAs in the oncogenesis of human colorectal cancer (CRC) remain to be elucidated. This study aimed to investigate the roles of *hsa_circ_0000523* and its parental gene *METTL3* in regulating cell proliferation, apoptosis and metastasis in a human CRC cell line (HCT116).

Methods: HCT116 cells were left untreated, transfected with *hsa_circ_0000523*- or *METTL3*-expressing plasmid, transfected with siRNA oligo against *hsa_circ_0000523* or *METTL3*, or transfected with negative control vector or siRNA oligo. All transfections were performed with Lipofectamine 2000. Transcriptional levels of *hsa_circ_0000523*, *miR-let-7b*, and *METTL3* were measured by real-time quantitative polymerase chain reaction. Cell proliferation was assessed by CCK-8 assay. Apoptosis was evaluated by flow cytometry after staining for annexin V and propidium iodide. Cellular potential for migration was detected by transwell assay.

Results: In HCT116 cells, *hsa_circ-0000523* indirectly regulated *METTL3* expression by suppressing the transcription of *miR-let-7b*. The expression of *METTL3* promoted cell proliferation and suppressed apoptosis. Higher levels of *METTL3* expression were associated with more aggressive tumor invasion.

Conclusion: The *hsa_circ_0000523/miR-let-7b/METLL3* axis functions in the tumorigenesis and pathogenesis of human CRC. Our results suggest that circRNAs and *METTL3* may be used for the highly sensitive diagnosis of CRC and predicting prognosis in patients who have undergone therapy.

Background

Colorectal cancer (CRC) is one of the most common malignancies worldwide[1, 2]. In year 2012, over 1 million new cases of CRC were diagnosed, accounting for approximately 10% of the global cancer burden[3]. Currently, CRC is the 4th most common cause of cancer-related mortality[4, 5]. Although conventional treatments such as surgery, chemotherapy, radiotherapy, and immunotherapy have significantly improved the survival of CRC patients, their mortality and rates of relapse remain high[5, 6]. Therefore, specific mechanism-based treatments are still needed.

Recently, circular RNAs (circRNAs) have gained wide attention as a class of potential biomarkers for the early detection of CRC[7]. Circular RNA is a newly discovered type of non-coding RNA, which is distinct from traditional linear RNA with 5' and 3' ends [8]. Circular RNAs are formed by exon skipping or back-splicing events, during which the 5 'end of an upstream exon is spliced together with the 3' end of the downstream exon to form a circular molecule[8, 9]. CircRNAs have been shown to play an important role in post-transcriptional regulation, by acting as miRNA sponges to competitively inhibit RNA/miRNA transcriptional regulation[10, 11].

The abnormal expression of circRNA is closely associated with various diseases, including CRC[12–14]. Jiang *et al.* identified a large set of circRNA with significantly differential expression in a primary colorectal cancer cell line (SW480) and a metastatic colorectal cancer cell line (SW620), relative to a normal colon cell line (NCM460) [15]. *Hsa_circ_000984* has been reported to promote colon cancer growth and metastasis by sponging miR-106b[16]. Moreover, Bachmayr-Heyda[17] *et al.* demonstrated a global reduction in cirRNA abundance in colorectal cancer cell lines and clinical colorectal cancer specimens, compared to normal tissues. The authors described five specific cirRNAs (circ0817, circ3203, circ6229, circ7374, and circ7780) and proposed a potentially negative correlation between global circular RNA abundance and cell proliferation.

Among 5 cirRNAs identified by Bachmayr-Heyda *et al.*, we were interested in circ6229 (also known as hsa_circ_0000523) and its corresponding gene/linear mRNA, *METTL3* (methyltransferase-like 3). As a major RNA N6-adenosine methyltransferase (m^6A), *METTL3* is widely implicated in mRNA biogenesis, decay, and translation control[18]. *METTL3* has recently been shown to promote the growth, survival, and invasion of many human cancers[19], such as lung cancer[20], hepatocellular carcinoma[21], breast cancer[22], and pancreatic cancer[23]. However, the exact role of *METTL3* in the tumorigenesis and pathogenesis of colorectal cancer is largely unknown.

HCT116 cells are a population of malignant cells isolated from the primary cell culture of a single human colonic carcinoma[24]. In this study, using HCT116 cells, we identified a negative correlation between expression levels of *hsa_circ_0000523* and *miR-let-7b*, which is considered to be a microRNA with tumor suppressor ability[25]. In HCT116 cells, the pattern of expression of *METTL3*, which may be a target gene of *miR-let-7b*, was similar to that of *hsa_circ_0000523*. Through transfection of *METLL3*-expressing plasmid and *METTL3*-specific siRNA oligo, we demonstrated that *METLL3* promoted the proliferation, apoptosis, and migration of HCT116 cells. Our work suggested a potential role for the *hsa_circ_0000523/miR-let-7b/METLL3* axis in the tumorigenesis and pathogenesis of human colorectal cancer.

Methods

Cell culture

The human CRC cell line HCT116 (3111C0001CCC000158, BMCR, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories; Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco Laboratories), 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA), and grown in a humidified atmosphere with 5% CO_2 at 37˚C. Cells were cultured in a 75-cm^2 flask or a 10-cm dish (Corning; New York, USA), with confluence maintained below 80%. After digestion with 0.25% (w/v) trypsin and 0.5 mM ethylenediaminetetraacetic acid (EDTA) (Gibco Laboratories), cells were passaged every 2-3 days with a subcultivation ratio of 1:3 to 1:8.

Plasmid constructs and siRNA oligos

The full-length cDNA for human *METTL3* (GenBank accession No. NM_019852.5) was obtained from the cDNA library of HCT116 cells by reverse transcription polymerase chain reaction (RT-PCR). The vector used to generate full-length wild-type *METTL3* and *hsa_circ_0000523* was mammalian expression vector pcDNA3.1 (Thermo Fisher Scientific). The *METTL3* coding sequence (CDS) or hsa_circ_0000523 fragment was subcloned into the pcDNA3.1 vector via KpnI and BamHI double-enzymatic sites. Positive clones were selected, and the plasmid expressing *METTL3* or *hsa_circ_0000523* was verified by sequencing. The siRNA oligos used in this study were designed and synthesized by Wuhan GeneCreate Biological Engineering Co., Ltd., Wuhan, China. The sequences for these siRNA oligos were as follows: siR-NC, sense 5'-GCUACUUCAGACGAGCAUUdTdT-3', siR-METTL3, sense 5'-GCUGCACUUCAGACGAAUUdTdT-3', siR-circ-NC; sense 5'- CAACAGAGCAAGAAGUAGAUdTdT-3', and siR-circ-0000523, sense 5'- CAACAGAGCAAGAAGAUCUAdTdT-3'.

Transfection of plasmids and siRNA oligos

HCT116 cells were transfected with the empty pcDNA3.1 vector, METTL3-expressing pcDNA3.1 vector (pcDNA3.1-METTL3), negative control siRNA oligo (siR-NC), *METTL3*-specific siRNA oligo (siR-METTL3), negative control oligo for *hsa_circ_0000523* (siR-circ-NC), or *circ-0000523*-specific siRNA oligo (siR-circ-0000523), using Lipofectamine 2000 reagent (Thermo Fisher Scientific), according to the manufacturer's instructions. HCT116 cells seeded in 6-well plates were maintained at confluence of 70% to 80% in complete culture medium. Then 2.5 μg plasmid or 100 pmol siRNA oligo was added to 250 μL Opti-MEM (Thermo Fisher Scientific), and 5 μL Lipofectamine 2000 reagent was added to 250 μL Opti-MEM. The diluted plasmid or siRNA oligo was mixed with diluted Lipofectamine 2000 reagent and kept at room temperature for 20 min. After 500 μL serum-free medium had been placed in each well, Opti-MEM mixture was added. After incubation of transfected cells at 37˚C for 4 h, the culture medium was changed to regular DMEM supplemented with 10% FBS.

RNA isolation and reverse transcription

Total RNA was extracted from cells with Trizol reagent (Thermo Fisher Scientific), according to the manufacturer's protocols. Briefly, after treatment, cell pellets were re-suspended in 1 mL Trizol and mixed well. Then, 0.2 mL of chloroform was added to the cell suspension, which was shaken vigorously for 15 s, then incubated for 3 min at room temperature. After centrifugation of the suspension at 12,000´g for 10 min at 4˚C, supernatant was collected and subjected to RNA precipitation by adding 0.5 mL of isopropanol at 4˚C. The RNA pellet was obtained by centrifugation at 12,000´*g* for 10 min at 4˚C, then washed with 75% ethanol. The appropriate amount of RNase-free distilled water was used to dissolve extracted RNA. The concentration of RNA was measured with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). For each sample, 1 μg of RNA was reverse-transcribed to cDNA with the ReverTra Ace qPCR RT Kit (catalogue number FSQ-101; Toyobo Co., Ltd.; Osaka, Japan), according to the specifications in the product manual.

Real-time quantitative PCR (RT-qPCR)

For RT-qPCR, SYBR Green Realtime PCR Master Mix (catalogue number QPK-212; Toyobo Co., Ltd.; Osaka, Japan) was used to measure the expression of targeted genes, according to the manufacturer's instructions. For quantification of *hsa_circ_0000523* and *let-7b*, *U6* was used as an internal reference gene. *GAPDH* was used as an internal reference gene for quantification of *METTL3*. The conditions for PCR were set as follows: pre-denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and elongation at 60°C for 30 s. The 7900HT Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific) was used to perform the assay. The $2^{-\Delta\Delta Ct}$ method was used to calculate differences between the experimental and control groups in expression of the target gene. The calculation formula was as follows: $\Delta\Delta Ct = \Delta Ct_{\text{experimental group}} - \Delta Ct_{\text{normal group}}$, $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{internal reference}}$. The sequences of primers used for RT-qPCR are presented in Table 1.

CCK-8 assay to measure cell proliferation

After transfection of empty pcDNA3.1 vector, METTL3-expressing pcDNA3.1 vector, siR-NC oligo, or siR-METTL3 oligo, HCT116 cells were seeded into flat-bottomed 96-well plates at a concentration of 3×10^3–6×10^3 cells per well, in 100 μL culture medium. Each treatment condition was replicated in 9 wells. Cells were incubated for 24 h, 48 h or 72 h in an incubator with 5% CO_2 at 37˚C. Cell proliferation was assayed with the CCK-8 assay (Beyotime Biotech Inc, Shanghai, China), according to the manufacturer's instructions. Briefly, 20 μL of 5 mg/mL CCK-8 reagent was added to the culture medium, and cells were further cultured for an additional 4 h. The optical density (OD) values of all wells were measured with a plate reader (Thermo Fisher Scientific) at 450 nm.

Cell apoptosis

HCT116 cells seeded in 6-well plates were transfected with empty pcDNA3.1 vector, METTL3-expressing pcDNA3.1 vector, siR-NC oligo, or siR-METTL3 oligo. At 48 h after transfection, cells were detached by incubation with 0.25% trypsin-EDTA solution (Gibco Laboratories), then harvested. Flow cytometric analysis was performed to detect apoptosis using the Annexin V-fluorescein isothiocyanate (FITC) /propidium iodide (PI) Apoptosis Kit (Beyotime Biotech Inc., Shanghai, China), according to the manufacturer's protocol. Briefly, after one wash in phosphate-buffered saline and one wash in binding buffer, cells were stained with Annexin V-FITC/PI for 20 min at room temperature, in the dark. After another wash in binding buffer, labeled cells were detected immediately by a flow cytometer (CytoFLEX S, Beckman Coulter; Brea, CA, USA). Data were analyzed with CytExpert Software (Beckman Coulter).

Cell migration

The cellular potential for migration was determined using a 24-well transwell plate with 8.0-μm Pore Polyester Membrane Inserts (Corning; Thermo Fisher Scientific). Matrigel (Corning) was melted overnight at 4˚C and diluted to a final concentration of 1 mg/mL in pre-cooled serum-free medium. Then, 100 mL of diluted matrigel was added to the bottom of the upper chamber. The plate was then incubated at 37˚C for

4-5 h to dry the matrigel. At 24 h after transfection, HCT116 cells in the logarithmic growth phase were seeded in triplicate at a density of 1×10^6 cells/well. Cells were seeded on top of the transwell plate, in 100 μL DMEM supplemented with 0.1% bovine serum albumin. Then 0.8 mL of DMEM supplemented with 10% FBS was added to the lower chamber as a chemoattractant. After 24 h of incubation, cells on the top surface of the insert were removed with a cotton swab. Cells that had migrated to the lower surface of the membrane were fixed with 4% paraformaldehyde, stained with 800 μL Giemsa solution (Beyotime Biotech Inc., Shanghai, China). Cells were visualized by a microscope (CKX-41; Olympus, Japan). Counts were obtained for three randomly selected optical fields.

Statistical analysis

All data were analyzed using SPSS Version 21.0 software (IBM Corp., USA). These data are presented as mean ± standard deviation (SD). Quantitative data were compared using the chi-square test. Comparisons between groups were performed with Student's t-test. $P < 0.05$ was accepted as an indication of statistical significance.

Results

Hsa_circ-0000523 regulates *METTL3* expression by modulating levels of *miR-let-7b* in HCT116 cells

To explore the role of *hsa_circ_0000523* circRNA in the tumorigenesis and pathogenesis of CRC, we established an *in vitro* model by manipulating the expression of *hsa_circ_0000523* in the HCT116 human CRC cell line. As shown in Figure 1A, the mammalian expression vector pcDNA3.1 mediated over-expression of *hsa_circ_0000523*. The transcription of hsa_circ_0000523 was increased by more than 20-fold in HCT116 cells, compared to cells transfected with empty vector. Efficient knockdown of *hsa_circ_0000523* was observed in HCT116 cells transfected with *hsa_circ_0000523*-specific siRNA oligo, which displayed a decrease in expression of *hsa_circ_0000523* of > 80%, compared with levels observed in HCT116 cells transfected with control siRNA oligo (Fig. 1A).

We then evaluated the impact of *hsa_circ_0000523* expression on the expression of *miR-let-7b*, a microRNA that may be a taret of *hsa_circ_0000523* in HCT116 cells. A strongly negative correlation between the expression of *hsa_circ_0000523* and *miR-let-7b* was observed. Among the various treatment conditions, HCT116 cells transfected with *hsa_circ_0000523*-expressing plasmid displayed the highest level of *hsa_circ_0000523* and the lowest level of *miR-let-7b*. Among all treatment groups, HCT116 cells with significant knockdown of *hsa_circ_0000523* had the highest expression of *miR-let-7b* (Fig. 1B). In HCT116 cells, expression of *METTL3*, one of the targets of *miR-let-7b*, was similar to that of *hsa_circ_0000523* (Fig. 1C). HCT116 cells with the highest levels of *hsa_circ_0000523* transcription also had the highest levels of *METTL3* mRNA. HCT116 cells with the lowest levels of *hsa_circ_0000523* transcription had the lowest levels of *METTL3* mRNA (Fig. 1C). An hsa_circ_0000523/miR-let-7b/METTL3 axis was thus identified in HCT116 cells.

Positive correlation between *METTL3* expression and cell proliferation in HCT116 cells

Because METTL3 is the endpoint effector molecule for the hsa_circ_0000523/miR-let-7b/METLL3 axis, we focused on elucidating the effects of *METTL3* expression on the proliferation, apoptosis, and migration of HCT116 cells. Similarly, HCT116 cells with over-expression or knockdown of *METTL3* were generated by transfection of METTL3-expressing pcDNA3.1 plasmid or *METTL3*-specific siRNA oligo, respectively. Levels of METTL3 mRNA were quantified by RT-qPCR. The results demonstrated that overexpression mediated by transfection with plasmid resulted in a > 200-fold increase in levels of *METTL3* transcription. Compared with untreated wild-type HCT116, siRNA oligo-transfection–mediated silence led to a > 70% decrease in levels of *METTL3* transcription (Fig. 2A).

After transfection with plasmids or siRNA oligos, HCT116 cells were further cultured for 24 h, 48 h, or 72 h. Cell proliferation was measured at these time-points using the CCK-8 assay. As expected, there was no significant difference in cell proliferation among untreated wild-type HCT116 cells, empty vector-transfected HCT116 cells, and negative control siRNA oligo-transfected HCT116 cells (Fig. 2B). Compared with the empty vector-transfected HCT116 cells, HCT116 cells with ectopic expression of METTL3 exhibited a significantly higher rate of proliferation at each time-point. Cell proliferation was decreased in HCT116 cells with knockdown of *METTL3* cells transfected with negative control siRNA oligos (Fig. 2B). Taken together, these results indicate a positive correlation between *METTL3* expression and the rate of cell proliferation in HCT116 cells.

METTL3 regulates apoptosis in HCT116 cells

We evaluated whether and how the expression of *METTL3* impacts apoptosis in HCT116 cells after transfection of plasmid or siRNA oligo with annexin V and PI staining. As expected, there was no significant difference on apoptotic rates among the untreated wild type HCT116 cells, empty vector-transfected HCT116 cells and negative control siRNA oligo-transfected HCT116 cells, all of which demonstrated an apoptotic rate of around 15% (Fig. 3A and Fig. 3B). The rate of apoptosis was significantly decreased in HCT116 cells with ectopic expression of *METTL3*, compared with HCT116 cells transfected with empty vector (12% vs, 15%). The rate of apoptosis was much higher in HCT116 cells with knockdown of *METTL3* than cells transfected with negative control siRNA oligos (20% vs. 15%; Fig. 3A and Fig. 3B). Therefore, an evidently negative correlation between *METTL3* expression and apoptotic rate was observed, which suggested that *METTL3* inhibited apoptosis in HCT116 cells.

Higher expression of *METTL3* is associated with more aggressive tumor invasion in HCT116 cells

To explore the role of METTL3 in regulating the metastasis of CRC, we performed transwell assays to examine the migration ability of HCT116 cells with various levels of *METTL3* expression. After plasmid or siRNA oligo transfection, HCT116 cells were seeded in the upper chamber of transwell plates, then cultured in medium containing a very low concentration of serum (0.5% BSA). The number of cells that had migrated to the surface of the lower chamber filled with DMEM containing 10% FBS can be used as an index for the metastatic ability of HCT116 cells. As shown by Giemsa staining of migrating cells in Figure 4A, more positively stained cells were observed in the group with over-expression of *METTL3*, compared with the group with knockdown of *METTL3*.

Statistical analysis confirmed the positive correlation between the expression of *METTL3* and the invasion abilities of HCT116 cells. As expected, there was no significant difference in migration ability among untreated wild-type HCT116 cells, empty vector-transfected HCT116 cells, and negative control siRNA oligo-transfected HCT116 cells (Fig. 4B). Compared with empty vector-transfected HCT116 cells, HCT116 cells with ectopic expression of *METTL3* exhibited significantly higher migration ability. Consistently, migration ability was decreased in HCT116 cells with knockdown of *METTL3*, compared with cells transfected with negative control siRNA oligos (Fig. 4A and Fig. 4B). These results suggest that higher expression of *METTL3* was associated with more aggressive tumor invasion in HCT116 cells.

Discussion

CircRNAs modulate the expression of parental genes by regulating alternative splicing or transcription and acting as competitive sponges for endogenous RNA or miRNA. CircRNAs may provide more comprehensive information on key genes involved in the oncogenesis and development of many cancers. They are potential accessible and noninvasive biological markers for the early detection of CRC. However, very few studies have reported on the specific functions of circRNAs in the tumorigenesis and pathogenesis of human CRC. In this study, we explored the expression patterns of *hsa_circ_0000523* and its parental gene *METTL3* in the human CRC cell line HCT116. Our results identified a potential *hsa_circ_0000523/miR-let-7b/METLL3* axis. The results of gain-of-function and loss-of-function assays showed that expression of *METTL3* promoted cell proliferation and migration and inhibited apoptosis in HCT116 cells.

Accumulating evidence has confirmed the abnormal expression and important biological functions of circRNAs in CRC[12–14]. Interestingly, the expression patterns of most cirRNAs identified by bioinformatics approaches have been reported to be significantly down-regulated in CRC cell lines and clinical CRC tissues[26]. Huang *et al.* demonstrated that the expression of *circ-ITCH* was much lower in CRC tissues, compared with adjacent noncancerous tissues[27]. *Cir-ITCH* was found to sponge tumorigenic *miR-7* and *miR-20a*, which contribute to the malignancy of CRC. A positive correlation between transcription of *circ-ITCH* and parental gene *ITCH* has been identified[27]. More importantly, expression of *circ-ITCH* was found to suppress cell proliferation in CRC cells[27]. Similarly, Zhu *et al.* reported that *circ-BANP* was expressed in 35 CRC tissues, and knock down of *circ-BANP* significantly decreased the proliferation of CRC cells[28].

We evaluated the expression levels of *hsa_circ_0000523*, its potential target *miR-let-7b*, and its parental gene *METTL3* in HCT116 cells with varied expression levels of *hsa_circ_0000523*. Similar to the expression patterns of *cir-ITCH* and *ITCH*, the transcriptional level of *hsa_circ_0000523* was positively associated with mRNA levels of linear *METTL3* in HCT116 cells. Although we did not measure the impacts of *hsa_circ_0000523* expression on cell proliferation, the ability of *METTL3* expression to increase HCT116 proliferation suggest a similar proliferative role for *hsa_circ_0000523*. However, a recent report from Jin *et al.* demonstrated that *hsa_circ_0000523* exerted anti-proliferative effects and promoted apoptosis in two other human CRC cell lines, SW480 and SW620[29]. Therefore, although

hsa_circ_0000523 can modulate the expression of METTL3 by acting on *miR-let-7b*, additional studies will be necessary to determine whether the expression of *METTL3* can significantly impact the transcription of *hsa_circ_0000523* in HCT116 cells.

CircRNA may regulate the proliferation of CRC by sequestering multiple miRNAs. For instance, circHIPK3 was shown to be over-expressed in CRC cells, compared with normal tissue, and regulates cell proliferation by sponging 9 miRNAs with 18 potential binding sites[30]. Specifically, circ-HIPK3 was shown to bind to and inhibit the activity of miR-124, a tumor suppressor usually down-regulated in CRC[31]. Therefore, circRNAs may modulate the tumorigenic proliferation of CRC cells by diminishing the antitumor effects of certain tumor suppressive miRNAs.

M*iR-let-7b*, shown here to be a potential target of *hsa_circ_0000523*, is also a tumor suppressor in multiple cancers[25]. Human *miR-let-7b* was found to be down-regulated in various cancers, and the induction of tumorigenesis can be inhibited in normal cells by ectopic expression of *miR-let-7b*[32, 33]. Human *miR-let-7* can inhibit cancer growth by targeting various oncogenes and inhibiting key regulators of several mitogenic pathways in cancer. These results point to the therapeutic potential of human *miR-let-7* in cancer therapy[25]. Therefore, high levels of *hsa_circ_0000523* and low levels of *miR-let-7* may contribute to tumorigenesis in CRC.

On the other hand, circRNA may regulate the proliferation of CRC by indirectly targeting oncogenes. In this study, *METTL3*, the parental gene of *hsa_circ_0000523*, and a target gene for *miR-let-7b*, was shown to promote the growth, survival, and invasion of human lung cancer[20]. Moreover, the oncogenic roles of *METTL3* have been demonstrated in many solid tumors and hematopoietic malignancies[34, 35]. To the best of our knowledge, this is the first report to describe the role of *METTL3* in CRC cells. The expression of *METTL3* in HCT116 cells was found to be positively correlated with cell proliferation and cancer invasion, but negatively correlated with apoptosis. These findings suggest that *METTL3* functions like an oncogene in CRC, possibly by promoting the translation of other oncogenes. In contrast, *METTL3* was found to be a tumor suppressor in renal cell carcinoma [36]. *METTL3* has been demonstrated to promote cell proliferation, migration, and invasion in two human renal cell carcinoma cell lines, CAKI-1 and CAKI-2. These effects are mediated by modulation of the epithelial-to-mesenchymal transition and PI3K-Akt-mTOR signaling [36]. The molecular mechanisms underlying the diverse roles of *METTL3* in renal cell carcinoma and CRC remain to be elucidated.

Conclusion

Through mammalian expression vector transfection-mediated gain-of-function studies and siRNA oligo transfection-mediated loss-of-function studies, we identified a potential *hsa_circ_0000523/miR-let-7b/METLL3* axis that contributes to tumorigenesis and pathogenesis in the human CRC cell line HCT116. METTL3 was shown to promote cell proliferation and migration and to inhibit apoptosis in HCT116 cells. Our work on the *hsa_circ_0000523/miR-let-7b/METLL3* axis may facilitate highly sensitive diagnosis of CRC and prognosis prediction in patients after therapy.

Declarations

Acknowledgements

We strongly appreciate discussions with colleagues in Professor Yun Zhu's laboratory. We thank the staff at Wuhan GeneCreate Biological Engineering Co., Ltd., Wuhan, China for technical help and advice on experiments.

Authors' Contributions

Conception and design: Yansheng Wang, Baolei Zhang, Li Li; Development of methodology: Yun Zhu, Yong Zhang, Hao Liu; Acquisition of data (acquisition and management of patients, facilities, etc.): Hao Liu, Tao Shen; Analysis and interpretation of data (e.g., statistical analysis, zsbiostatistics, computational analysis): Yun Zhu, Hao Liu; Writing, review, and/or revision of the manuscript: Yansheng Wang, Baolei Zhang; Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Yansheng Wang, Yirong Teng; Study supervision: Hao Liu, Yirong Teng.

Funding

This work was supported by grants from the Scientific research fund project of Yunnan Education Department (grant number 2019J1306) ; by grants from the National Natural Science Foundation of China (grant numbers 81660156, 81760136) and the Joint Special Fund of Applied Fundamental Research of Kunming Medical University and the Science and Technology Office of Yunnan Province (grant numbers 2018FE001(-174), 2018FE001(-175); by grants from Yunnan Special Funds for training high-level health and family planning technical personnel, grant no. H-2017064; H-2017028. The funders had no role in study design, data collection, data analysis, decision to publish, or preparation of the manuscript.

Consent for publication

We have obtained consents to publish this paper from all the participants of this study.

Availability of data and materials

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

Conflict of interest

The authors have declared that no competing interests exist.

Competing interests

All authors declare no competing interests.

Ethics approval and consent to participate

Not applicable

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Tables

Table 1
The sequences of primers used for RT-qPCR in this study.

Genes	Primer sequences (5’ – 3’)
hsa_circ-0000523	F: CAGCATCGGAACCAGCAAAG
	R: CTGGGCTGTCACTACGGAAG
miR-let-7b	F: GGGTGAGGTAGTAGGTTGT
	R: CAGTGCGTGTCGTGGAGT
U6	F: CTCGCTTCGGCAGCACA
	R: AACGCTTCACGAATTTGCGT
METTL3	F: GTGTCGGAGGTGATTCCAGT
	R: CTGCGCATCTCATCATCTGT
GAPDH	F: GTCAGTGGTGGACCTGACCT
	R: TGCTGTAGCCAAATTCGTTG

F, forward; R, reverse.

Figures

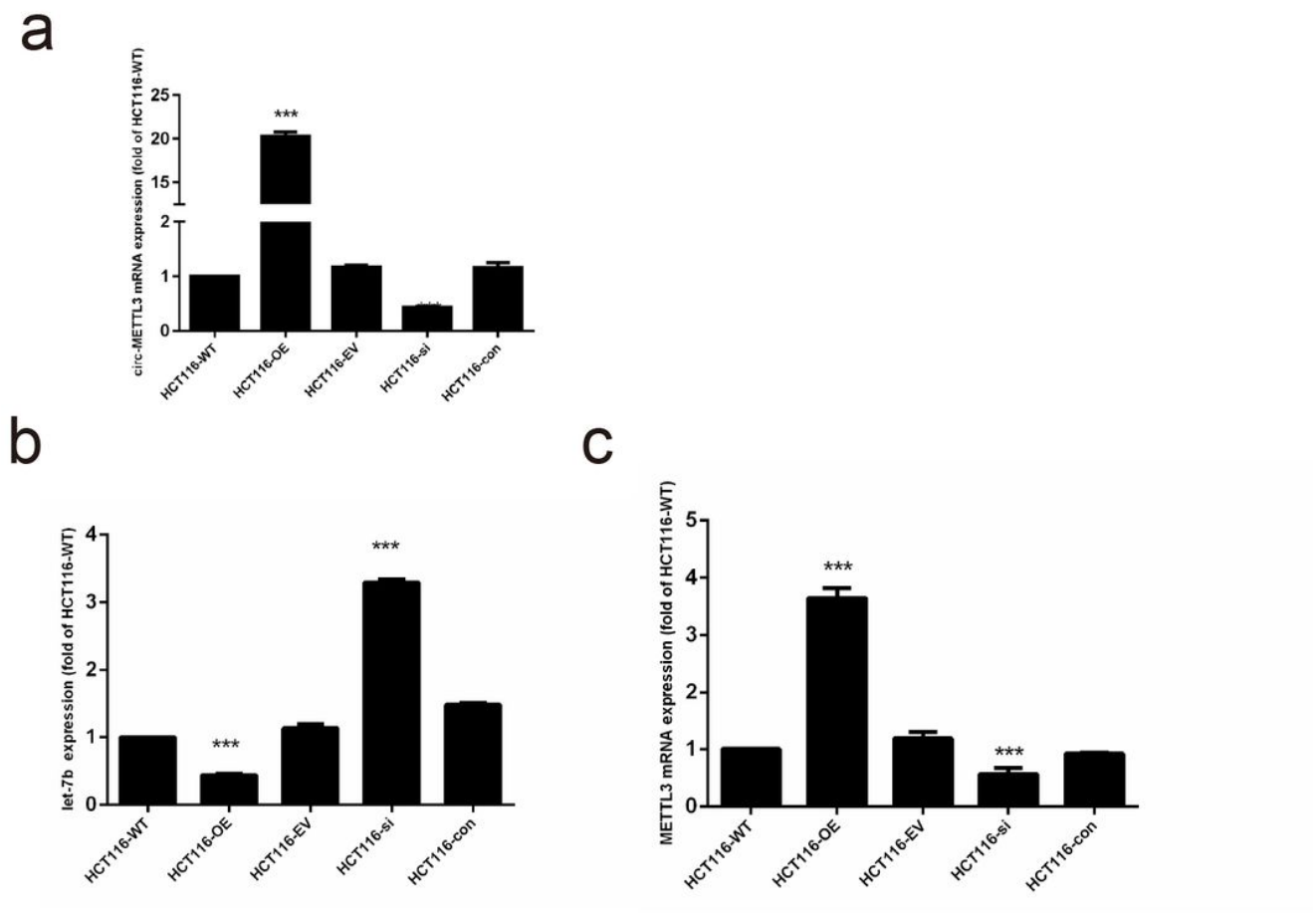

Figure 1

Hsa_circ-0000523 indirectly regulated METTL3 expression by suppressing transcription of miR-let-7b in HCT116 cells. a-c HCT116 cells were left untreated or transfected with hsa_circ-0000523-expressing plasmid, empty pcDNA3.1 vector plasmid, hsa_circ-0000523-specific siRNA oligo, or control siRNA oligo. At 48 h after transfection, HCT116 cells were harvested, and transcriptional levels of hsa_circ-0000523 (a), miR-let-7b (b) and METTL3 (c) were quantified by RT-qPCR. WT, untreated wild-type HCT116 cells; OE, HCT cells with over-expression of hsa_circ-0000523; EV, HCT116 cells transfected with empty vector; si, HCT116 cells transfected with hsa_circ-0000523-specific siRNA oligo; con, HCT116 cells transfected with negative control siRNA oligo. n = 3 for each group; *P < 0.05, **P < 0.01, ***P < 0.001, compared with the WT group.

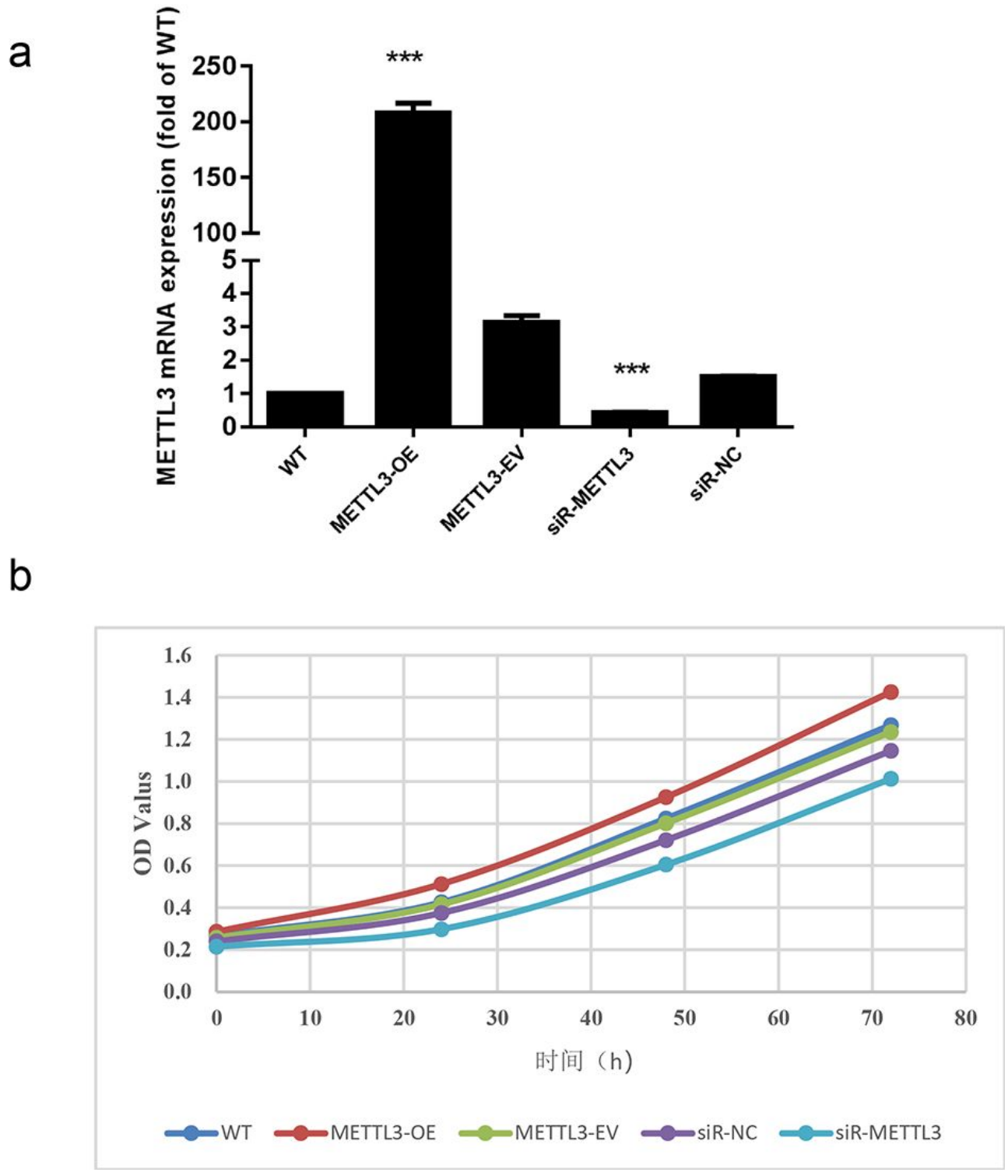

Figure 2

Expression of METTL3 promoted cell proliferation in HCT116 cells. HCT116 cells were left untreated or transfected with METTL3-expressing plasmid, empty pcDNA3.1 vector plasmid, METTL3-specific siRNA oligo, or control siRNA oligo. a At 48 h after transfection, cells were harvested, and mRNA levels of METTL3 were quantified by RT-qPCR. b Cell proliferation was measured with the CCK-8 method at 24 h, 48 h, and 72 h after transfection. WT, untreated wild-type HCT116 cells; METTL3-OE, HCT cells with over-

expression of METTL3; METTL3-EV, HCT116 cells transfected with empty vector; siR-METTL3, HCT116 cells transfected with METTL3-specific siRNA oligo; siR-NC, HCT116 cells transfected with negative control siRNA oligo. n = 3 for each group; *P < 0.05, **P < 0.01, ***P < 0.001, compared with the WT group.

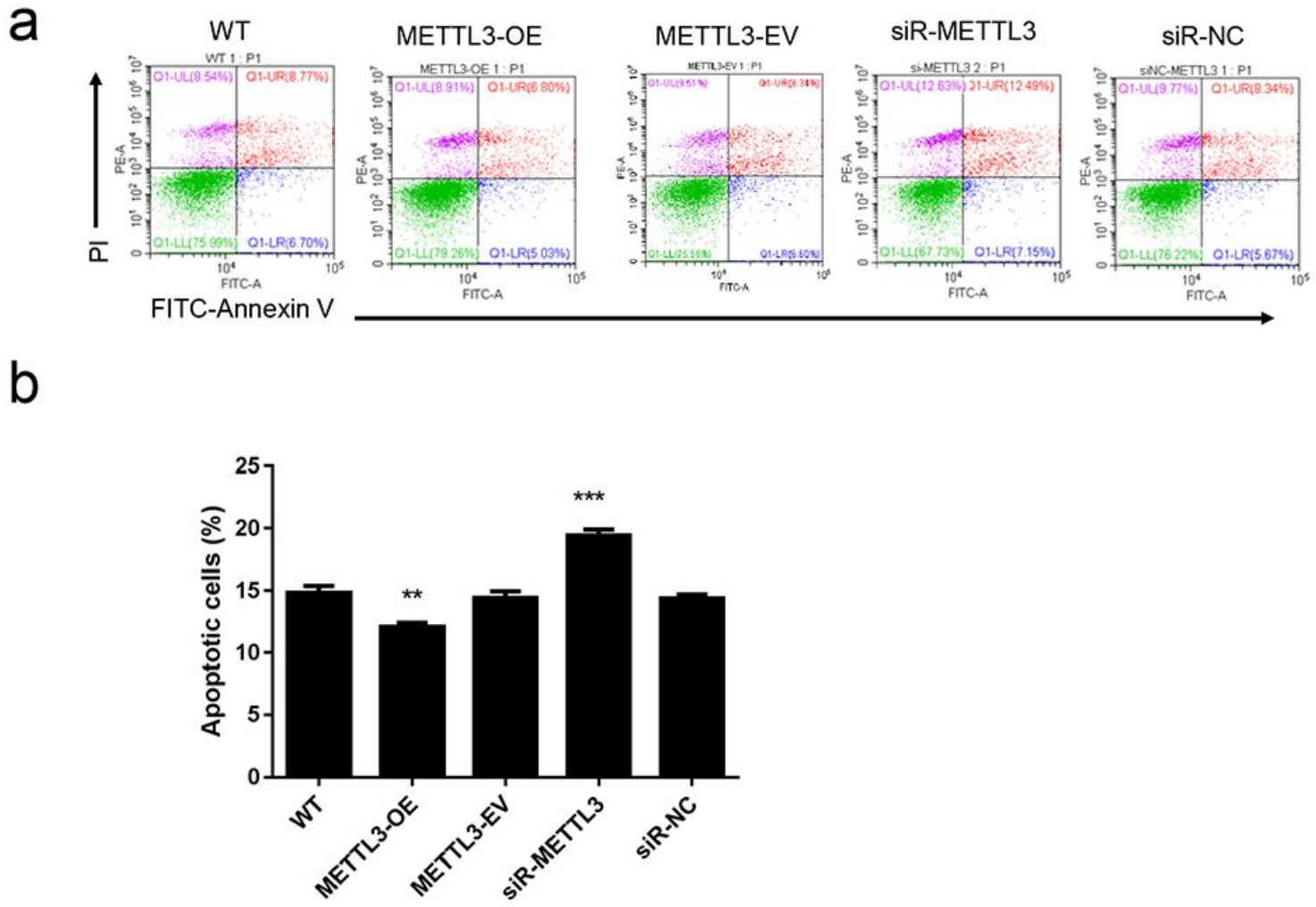

Figure 3

Expression of METTL3 suppressed apoptosis in HCT116 cells. HCT116 cells were treated as described in Figure 2. At 48 h after transfection, levels of apoptosis were assessed with annexin V-FITC/PI staining. a Representative flow cytometric profiles show staining for annexin V and PI in HCT116 cells after the indicated treatments. b Summarized data show the rate of apoptosis in HCT116 cells after the indicated treatments. Annexin V-positive cells were considered to be apoptotic cells. n = 3 for each group; ***P < 0.001, compared with the WT group.

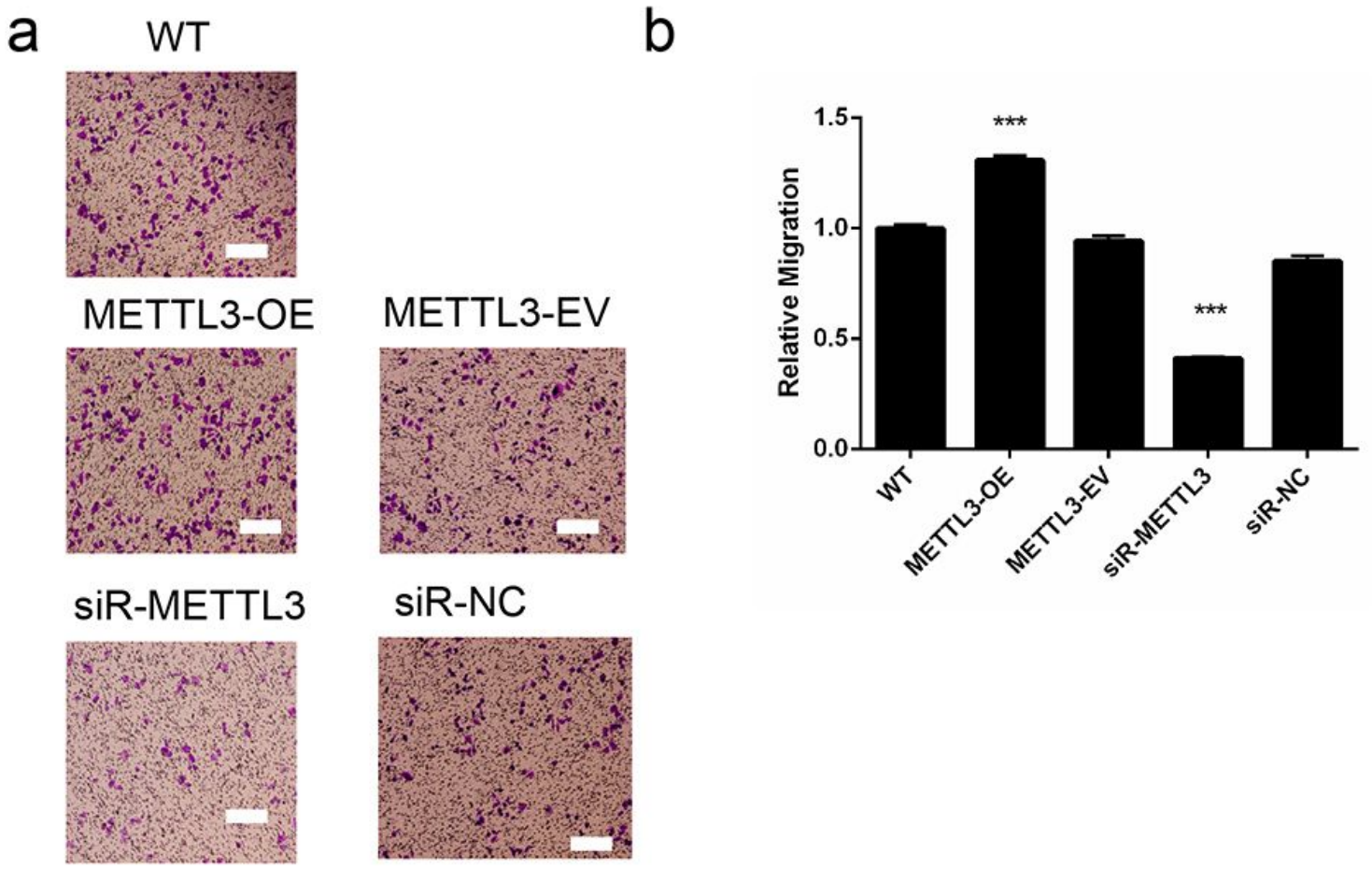

Figure 4

In HCT116 cells, higher levels of METTL3 were associated with more aggressive tumor invasion. HCT116 cells were treated as described in Figure 2. At 24 h after transfection, HCT116 cells in the logarithmic growth phase were seeded in the upper chamber of the transwell plate. After cells were incubated for another 24 h, the numbers of cells that had migrated to the surface of the lower chamber was measured with Giemsa staining. a Representative images show the results of Giemsa staining in HCT116 cells after the indicated treatments. Magnification, 100×. b Summarized data show the relative migration values of HCT116 cells after the indicated treatments. The relative migration value of untreated wild-type HCT116 (WT group) was arbitrarily set as 1. n = 3 for each group; *P < 0.05, **P < 0.01, ***P < 0.001, compared with the WT group.