Molecular and Cellular Biochemistry (2019) 457:83–91
https://doi.org/10.1007/s11010-019-03514-x

Activation of EMT in colorectal cancer by MTDH/NF-κB p65 pathway

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Received: 9 November 2018 / Accepted: 23 February 2019 / Published online: 1 March 2019

Abstract

Epithelial-mesenchymal transition (EMT) leads to tumor dissemination and metastasis. Metadherin (MTDH) is an oncogene that plays an important role in metastasis regulation. This study tries to investigate the effect of MTDH gene up-regulation on the activation of EMT in colorectal cancer (CRC) cells and identify the role of NF-κB p65. The CaCO2 cells were divided into three groups: one control group of cultured CaCO2 cells (C1), and two groups of CaCO2 cells co-transfected using human MTDH expression plasmid with either siRNA targeting human NF-κB p65 or its negative control (C2 and C3 respectively). The gene modification was confirmed by qPCR and the effect of gene modification on CRC aggravation was studied. MTDH up-regulation significantly promoted CRC cell proliferation, activated anaerobic respiration (glucose consumption and lactate production), and increased gene expression of multidrug resistance gene (MDR1), Snail transcription factor and NF-κB p65, but decreased the gene expression of E-cadherin. Moreover, MTDH up-regulation led to a significant increase in the acquisition of surface markers of CRC stem cells. Interference with NF-κB p65 gene expression reversed the action of MTDH gene up-regulation on MDR1 and E-cadherin gene expression and anaerobic respiration. Moreover, NF-κB p65 interference significantly decreased MTDH-induced cell proliferation and acquisition of surface markers of CRC stem cells but didn't affect the Snail transcription factor. MTDH-dependent EMT in CRC is activated via NF-κB p65 and is mediated by up-regulation of Snail. These results identify a pathway by which MTDH regulates NF-κB p65 induced EMT during CRC cell metastasis.

Keywords MTDH · NF-κB P65 · E-cadherin · Snail · EMT

Introduction

Colorectal cancer (CRC) is a major cause of mortality and morbidity worldwide. Numerous patients with CRC develop metastasis, like lymph node metastasis and distant metastasis, resulting in high overall mortality rate [1]. Cancer metastasis accounts for about 90% of cancer deaths [2].

The epithelial-mesenchymal transition (EMT) is the conversion of polarized epithelial cells to motile mesenchymal cells [3]. The EMT has a significant role in tumor cell invasion and migration. During cancer development, EMT leads to metastatic spread and tumor dissemination [2].

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Nuclear factor kappa B (NF-κB) comprises a family of inducible transcription factors that act as regulators of the host immune and inflammatory response [4]. NF-κB plays a significant role in multiple cellular processes including survival, proliferation and tumor cell metastasis. In unstimulated cells, NF-κB heterodimers p65 and p50 are sequestered in the cytosol by the inhibitor of κB (IκBs) [5]. The IκB kinase complex phosphorylates IκBs the phosphorylation make IκBs targeted for ubiquitination and subsequent degradation by the proteasome. Free p50/p65 heterodimers transfer to the nucleus and bind to the DNA to activate transcription of NF-κB-dependent genes [6].

Metadherin (MTDH) also known as astrocyte-elevated gene-1 (AEG-1), is an important oncogene. Overexpression of MTDH has been observed in different types of cancer, including breast, ovarian, prostate, lung, esophageal, and CRC [7–10].

Overexpression of MTDH could promote the invasion ability of human breast cancer cells by inducing EMT and NF-κB signaling pathway was involved in MTDH-induced

EMT of breast cancer cell in vitro [11]. Several evidences have suggested that MTDH activates NF-κB via degradation of IκBα and direct binding with p65 subunit [12].

MTDH was overexpressed in colon cancer and associated with the prognosis [13]. MTDH expression was significantly up-regulated gradually from normal mucosal cells to primary CRC, and finally to lymph node and liver metastasis. There was significant connection of MTDH gene expression with tumor location and stage in CRC [12].

It has been shown that MTDH is a key mediator of migration, invasion, and treatment resistance [14, 15]. Although several signaling pathways, including Ha-ras, phosphatidylinositol-3-kinases (PI3K)/Akt, NF-κB, and Wnt/beta-catenin, were found to be implicated in the function of MTDH, the precise mechanism of MTDH in invasion and metastasis in CRC is largely unknown [16].

The aim of the current study was to investigate the effect of MTDH up-regulation on the EMT of CRC cells and identify the role of NF-κB p65 in the pathway of activation of EMT by MTDH gene up-regulation in vitro.

Materials and methods

Cell line

The CaCO2 cell line was obtained from Vacsera, Egypt. The cell line was cultured in 75 cm^2 tissue culture flasks at 37 °C and 5% CO_2 in DMEM with L-glutamine (Lonza, USA), supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, USA) and 1% of a penicillin/streptomycin (Sigma-Aldrich, USA). Cells were harvested when 70–80% confluence was achieved [17].

Transient transfection

24 h before transfection, CaCO2 cells were seeded in 24-well plates containing 500 µL of serum-free DMEM with L-glutamine at a density of 2×10^5 cell per well. The CaCO2 cells were divided into three groups: one control group of cultured CaCO2 cells (C1), and two groups of CaCO2 cells co-transfected by human MTDH expression plasmid (Origen, USA) with either siRNA targeting human NF-κB p65 or its negative control (Santa Cruz/Biotechnology, Europe) (C2 and C3 respectively). The transfection was performed using 1 µL Lipofectamine 2000 reagent (Invitrogen, USA), 250 ng plasmid DNA, and 7.5 pmol of siRNA per well according to manufactured protocol. Transfected cells were incubated for 2 days at 37 °C and 5% CO_2 before gene modification analysis [18].

RNA extraction and preparation of cDNA

RNA-spin™ total RNA extraction kit (Intron Biotechnology, Korea) was used for total RNA extraction according to the manufacturer's instructions. NanoDrop (Denovix, USA) was used for measuring the concentration and purity of extracted RNA. For the RT-PCR, the Power cDNA synthesis kit (Intron Biotechnology, Korea) was used. According to the manufacturer's instructions, 2 µg of extracted RNA was reverse transcribed by AMV reverse transcriptase with an RNase inhibitor, and the final volume was adjusted to 20 µL. The program was the following: 42 °C for 60 min followed by inactivation at 70 °C for 5 min (Thermo cycler Biometra, USA).

Real-time quantitative polymerase chain reaction (qPCR)

Quantitative real-time PCR was carried out using the Realmod™ GH green real-time PCR master mix kit (Intron Biotechnology, Korea). The relative gene expression level of MTDH, Snail, E-cadherin, multidrug resistance gene (MDR1), and NF-κB p65 were determined by a real-time PCR system (Thermo Fisher Scientific, USA). The amplification program was the following: initial activation step 94 °C for 5 min followed by 40 cycles of denaturation 94 °C for 15 s, annealing/extension 59 °C for 50 s. The relative gene expression was normalized to GAPDH, and the fold difference in the expression of the target gene was calculated using Livak method ($2^{-\Delta\Delta C_T}$ method). All samples were done in triplicates [19].

The Primer3 plus software was used for primer design as we discussed in previous work (not published yet) (Table 1). The primers were purchased from (Biosearch technologies, USA).

Measurements of glucose and lactate

Glucose consumption and lactate production were measured as an indicator of the rate of anaerobic glycolysis [20]. After transfection experiments, the medium was collected for analysis of glucose and lactate levels. Glucose was measured spectrophotometrically using glucose kit (Biodiagnostic, Egypt). Lactate was measured colorimetrically using Cobas 6000 analyzer series (Roche/Hitachi Cobas c system, USA) by lactate Gin.2 kit (Roche Diagnostics, USA).

Cell proliferation assay

Methylthiazolyl blue tetrazolium (MTT; Thermofissure, USA) spectrophotometric dye assay was used to observe

Table 1 Primer sequence for real-time PCR

Gene (human)	Forward primer	Reverse primer
MTDH	TCTGCTGATCCCAACTCTGA	GAGCTCCCTCTCCCTTTTCT
MDR1	TGATTGCATTTGGAGGACAA	CCAGAAGGCCAGAGCATAAG
E-cadherin	GAACGCATTGCCACATACAC	AGCACCTTCCATGACAGACC
Snail	CTCTAGGCCCTGGCTGCTAC	GCCTGGCACTGGTACTTCTT
NF-κB p65	CTGGGAATCCAGTGTGTGAA	CACTGTCACCTGGAAGCAGA
GAPDH	TCACCAGGGCTGCTTTTAAC	GACAAGCTTCCCGTTCTCAG

MTDH metadherin, *MDR1* multidrug resistance gene, *NF-κB p65* nuclear factor kappa B p65, *GAPDH* glyceraldehyde phosphate dehydrogenase

cell proliferation ability. CaCO2 cells were plated in 96-well plates containing 100 µL serum-free RPMI 1640 without L-glutamine and phenol red media (Lonza, USA) at a density of 1×10^4 cells/well and allowed to grow for 24 h before transfection. After transfection experiments, cell proliferation was measured by incubating the cells for 4 h in 10 µL MTT solution (5 mg/mL) at 37 °C. For dissolving the formazan crystals, 150 µL dimethyl sulfoxide was added into each well, the absorbance was measured at 545 nm by ELISA microplate reader (Tecan, Austria).

Flow cytometry

According to Janakiram et al. [21] transfected cells were washed with phosphate-buffered saline (PBS) and then harvested and suspended in fluorescence-activated cell sorting (FACS) buffer (5×10^4 cells/100 µL). 10 µL of fluorochrome-conjugated anti-human CD44 and CD24 or the respective isotype controls were used to label the cell and incubated in the dark at 4 °C for 10 min. FACS buffer was used to wash the labeled cells, and the cells were processed by FACSCalibur (Becton Dickinson, USA). All antibodies were obtained from Miltenyi Biotec, Germany.

ELISA analysis of NF-κB p65

After trypsinization and washing with PBS, transfected cells were resuspended in PBS (pH 7.2–7.4) at a concentration of 1×10^6 cells/mL. The cell lysate was prepared by five repeated freezing-thawing cycles (freezing at − 80 °C for 5 min followed by thawing at 60 °C for 15 min) then centrifuged at 2000 rpm for 20 min and the supernatant was collected, and stored at − 80 °C until analysis was carried out by ELISA. NF-κB p65 was quantified using ELISA kits (Sunred Biotechnology, Germany) according to the manufacturer's instructions and OD was measured at 450 nm by ELISA microplate reader (Tecan, Austria). The sample concentration of NF-κB p65 was obtained from the standard curve and was expressed as ng/mL.

Statistical analysis

Numerical data were presented as mean ± SEM. The correlations between measured values were evaluated by Pearson's correlation coefficient. Comparisons among group were performed using one-way analysis of variance (ANOVA). All statistical analyses were carried out using the SPSS 18.0 statistical software. $P < 0.05$ was considered statistically significant.

Results

MTDH up-regulation in CRC cell line with or without NF-κB p65 interference

To identify the role of NF-κB p65 in the pathway of MTDH-induced EMT, the CaCO2 cells were divided into three groups: one control group of cultured CaCO2 cells (C1), and two groups of CaCO2 cells co-transfected using human MTDH expression plasmid with either siRNA targeting human NF-κB p65 or its negative control (C2 and C3 respectively). The MTDH overexpression and NF-κB p65 interference were confirmed by determination of gene expression of MTDH and NF-κB p65 by real-time PCR. After co-transfection, the gene expression of MTDH in NF-κB p65 silenced/MTDH-overexpressed CaCO2 cells (C2) and MTDH-overexpressed CaCO2 cells (C3) increased significantly to about 5.381 and 5.657 fold of the amount expressed by cultured CaCO2 cells (C1) respectively. In addition to that the gene expression of NF-κB p65 in MTDH-overexpressed CaCO2 cells (C3) was increased significantly to about 4.205 fold of the amount expressed by the cultured CaCO2 cells (C1), while the gene expression of NF-κB p65 in silenced/MTDH-overexpressed CaCO2 cells (C2) showed no significant difference from cultured CaCO2 cells (C1) (Fig. 1a).

Also, the silencing of NF-κB p65 gene expression was confirmed by determination of the protein level of NF-κB p65 using ELISA technique. NF-κB p65 protein level increased significantly in MTDH-overexpressed CaCO2 cells (C3) (5.676 ± 0.086 ng/mL) compared to cultured

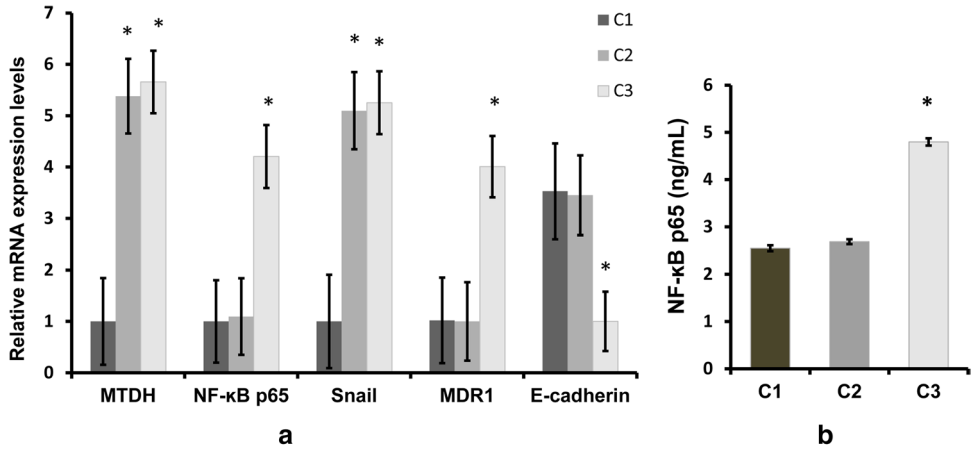

Fig. 1 **a** Gene expression analysis of CaCO2 cells after MTDH up-regulation with or without NF-κB p65 interference. Data are presented as mean of relative gene expression ± relative error. **b** Biochemical assay showing a significant decrease in NF-κB p65 protein level after interference with NF-κB p65 in MTDH-overexpressed CaCO2 cells. Data are presented as mean ± SEM. *Significant versus the control group ($P < 0.05$). C1: CaCO2 cells (control). C2: NF-κB p65 silenced/MTDH-overexpressed CaCO2 cells. C3: MTDH-overexpressed CaCO2 cells. MTDH: metadherin. MDR1: multidrug resistance gene 1. NF- κB p65: nuclear factor kappa B p65

CaCO2 cells (C1) (2.552 ± 0.056 ng/mL). On the other hand, NF-κB p65 protein level decreased significantly in NF-κB p65 silenced/MTDH-overexpressed CaCO2 cells (C2) (2.691 ± 0.052 ng/mL) compared to MTDH-overexpressed CaCO2 cells (C3) (Fig. 1b).

Interference with NF-κB p65 gene expression reversed the action of MTDH gene up-regulation on EMT

Snail and Slug transcription factors inhibit E-cadherin transcription, and increments in these repressors are tightly connected with EMT during cancer growth and invasion [22]. Hence, we examined the expression of Snail as a transcriptional repressor. After MTDH up-regulation, the gene expression of Snail transcription factor in NF-κB p65 silenced/MTDH-overexpressed CaCO2 cells (C2) and MTDH-overexpressed CaCO2 cells (C3) increased significantly to about 5.098 and 5.252 fold respectively of the amount expressed by cultured CaCO2 cells (C1). Moreover, the gene expression of Snail transcription factor in NF-κB p65 silenced/MTDH-overexpressed CaCO2 cells (C2) showed no significant difference from the MTDH-overexpressed CaCO2 cells (C3).

Our result showed that MTDH gene up-regulation in MTDH-overexpressed CaCO2 cells (C3) significantly decreased the epithelial marker E-cadherin compared to cultured CaCO2 cells (C1). On the other hand, the interference with the gene expression of NF-κB p65 in MTDH-overexpressed CaCO2 cells significantly increased the gene expression of E-cadherin. The amount of gene expression of E-cadherin was significantly higher in cultured CaCO2 cells (C1), and NF-κB p65 silenced/MTDH-overexpressed CaCO2 cells (C2) than the amount expressed by MTDH-overexpressed CaCO2 cells (C3) (3.531 and 3.453 folds respectively). Also, the amount of E-cadherin expressed by NF-κB p65 silenced/MTDH-overexpressed CaCO2 cells (C2) showed no significant difference from that expressed by cultured CaCO2 cells (C1) (Fig. 1a).

Interference with NF-κB p65 gene expression reversed the action of MTDH gene up-regulation on MDR1

Our result showed that MTDH gene up-regulation in MTDH-overexpressed CaCO2 cells (C3) significantly increased the gene expression of MDR1 (responsible for drug resistance) compared to cultured CaCO2 cells (C1). On the other hand, the interference with the gene expression of NF-κB p65 in MTDH-overexpressed CaCO2 cells significantly decreased the gene expression of MDR1. The amount of MDR1 gene expressed by MTDH-overexpressed CaCO2 cells (C3) was significantly higher than that expressed by NF-κB p65 silenced/MTDH-overexpressed CaCO2 cells (C2) (4.008 fold). Also, the amount of MDR1 expressed by NF-κB p65 silenced/MTDH-overexpressed CaCO2 cells (C2) showed no significant difference from that expressed by cultured CaCO2 cells (C1) (Fig. 1a).

Interference with NF-κB p65 gene expression reversed the action of MTDH gene up-regulation on the rate of anaerobic glycolysis

Glucose consumption and lactate production were measured as an indicator of the rate of anaerobic glycolysis. MTDH gene up-regulation significantly increase glucose consumption and lactate production in MTDH-overexpressed CaCO2 cells (C3) (25.211 ± 1.099 mg/dL, 23.563 ± 0.882 mg/dL respectively) compared to cultured CaCO2 cells (C1) (9.301 ± 0. 253 mg/dL, 9.500 ± 0.218 mg/dL respectively). On the other hand, NF-κB p65 silenced/MTDH-overexpressed CaCO2 cells (C2) showed no significant difference in glucose consumption and lactate production from cultured CaCO2 cells (9.558 ± 0.786 mg/dL, 9.450 ± 0.805 mg/dL respectively). Therefore, the interference with the gene expression of NF-κB p65 in MTDH-overexpressed CaCO2 cells significantly decreased MTDH-induced glucose consumption and lactate production (Fig. 2a).

Interference with NF-κB p65 gene expression reversed the action of MTDH gene up-regulation on cell proliferation rate

MTT proliferation assay test showed that MTDH up-regulation significantly increased the survival rate of MTDH-overexpressed cells compared to cultured CaCO2 cells (174.903 ± 2.383%). On the other hand, the cell proliferation rate of NF-κB p65 silenced/MTDH-overexpressed CaCO2 cells (C2) showed no significant difference from cultured CaCO2 cells (97.471 ± 1.933%). Therefore, the interference with NF-κB p65 in MTDH-overexpressed CaCO2 cells significantly decreased MTDH-induced cell proliferation (Fig. 2b).

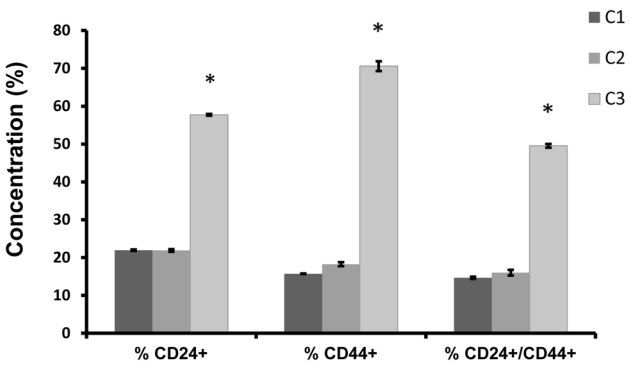

Fig. 3 NF-κB p65 gene silencing reversed the action of MTDH gene up-regulation on surface markers that characterize CRC stem cells. Data are presented as mean ± SEM. *Significant increase versus the control group ($P < 0.05$). C1: CaCO2 cells (control). C2: NF-κB p65 silenced/MTDH-overexpressed CaCO2 cells. C3: MTDH-overexpressed CaCO2 cells

Interference with NF-κB p65 gene expression reversed the action of MTDH gene up-regulation on the percent of the colorectal cancer stem cell

The percent of cells acquired cancer stem cell markers ($CD24^+$, $CD44^+$ and $CD24^+/CD44^+$) increased significantly in MTDH-overexpressed CaCO2 cells (57.733 ± 0.233%, 70.600 ± 1.290%, and 49.533 ± 0.504%, respectively) compared to CaCO2 cells (21.967 ± 0.272%, 15.733 ± 0.120%, and14.653 ± 0.404%, respectively). While, the percent of cells acquired cancer stem cell markers in NF-κB p65 silenced/MTDH-overexpressed CaCO2 cells (C2) showed no significant difference from cultured CaCO2 cells (21.900 ± 0.346%, 18.267 ± 0.498%, and 16.020 ± 0.754%, respectively), Therefore, the interference with NF-κB p65 in MTDH-overexpressed CaCO2 cells significantly decreased MTDH-induced increment in CRC stem cells (Figs. 3, 4).

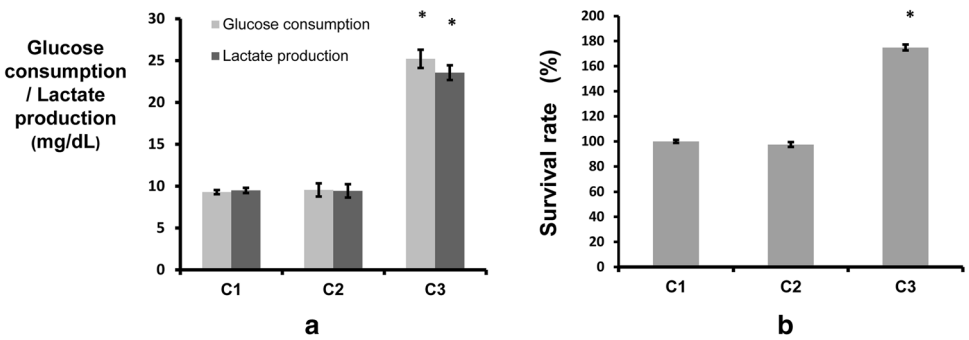

Fig. 2 **a** Biochemical assay showing that NF-κB p65 gene silencing reversed the action of MTDH gene up-regulation on the rate of anaerobic glycolysis. **b** NF-κB p65 gene silencing reversed the action of MTDH gene up-regulation on the survival rate of CaCO2 cells. Data are presented as mean ± SEM. *Significant increase versus the control group ($P < 0.05$). C1: CaCO2 cells (control). C2: NF-κB p65 silenced/MTDH-overexpressed CaCO2 cells. C3: MTDH-overexpressed CaCO2 cells

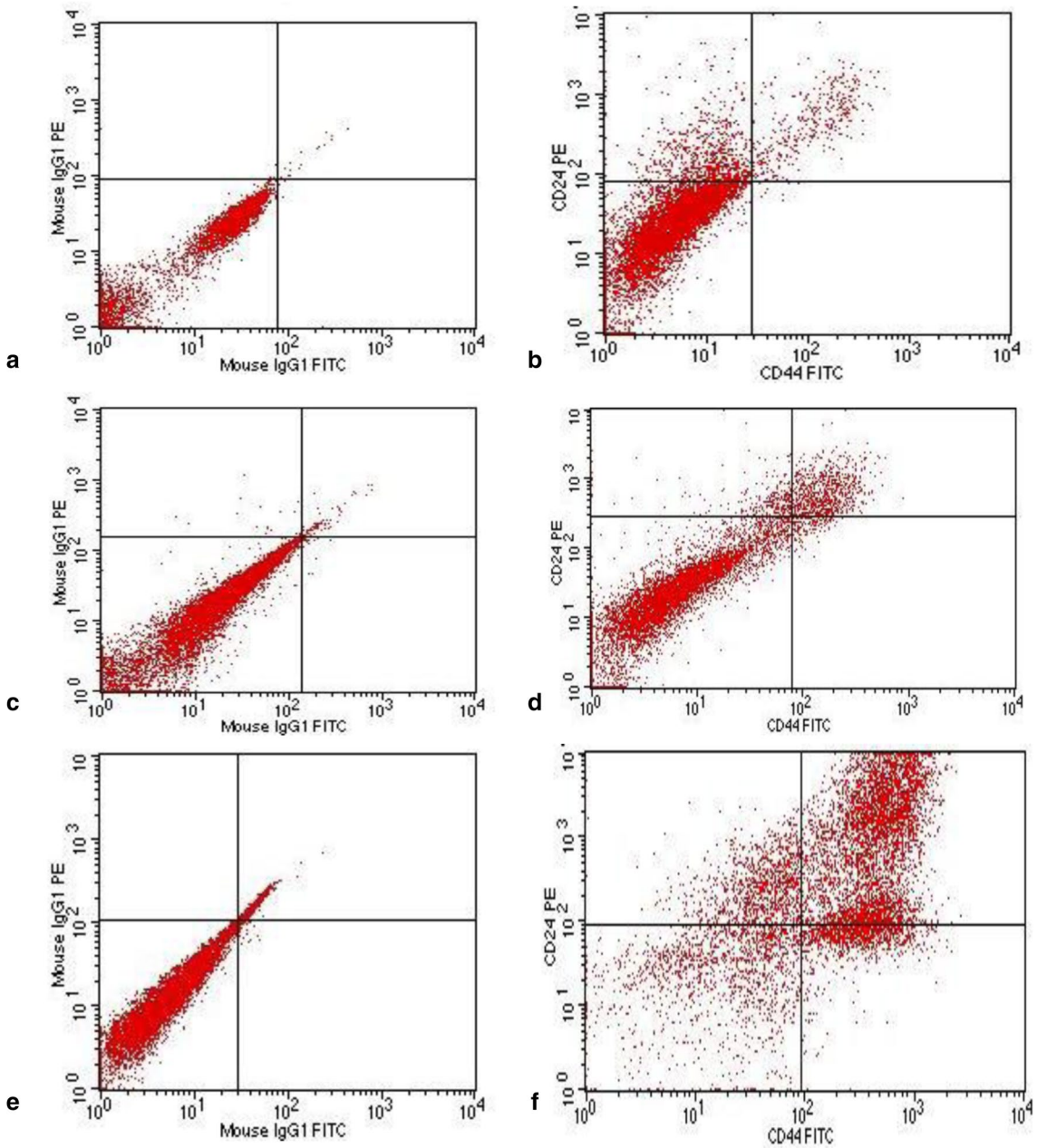

Fig. 4 Flow cytometric dot plot showing that NF-κB p65 gene silencing reversed the action of MTDH gene up-regulation on the acquisition of CRC stem cells surface markers. **a, c, e** Isotype negative control. **b, d, f** CD44/24 expression pattern of CaCO2 cells (C1), NF-κB p65 silenced/MTDH-overexpressed CaCO2 cells (C2) and MTDH-overexpressed CaCO2 cells (C3) respectively. The results shown are representative of three independent experiments

Correlation results

NF-κB p65 showed a significant positive correlation with each of glucose consumption ($r=0.960$, $P<0.001$), lactate production ($r=0.951$, $P<0.001$), percent of $CD24^+$ cells ($r=0.995$, $P<0.001$), percent of $CD44^+$ cells ($r=0.997$, $P<0.001$), percent of ($CD24^+/CD44^+$) cells ($r=0.994$, $P<0.001$), and cell proliferation ability ($r=0.989$, $P<0.001$).

Glucose consumption was also significantly positively correlated with each of lactate production ($r=0.936$, $P<0.001$), percent of $CD24^+$ cells ($r=0.970$, $P<0.001$), percent of $CD44^+$ cells ($r=0.963$, $P<0.001$), percent of ($CD24^+/CD44^+$) cells ($r=0.963$, $P<0.001$), and cell proliferation ability ($r=0.981$, $P<0.001$).

Lactate production was also significantly positively correlated with each of percent of $CD24^+$ cells ($r=0.985$, $P<0.001$), percent of $CD44^+$ cells ($r=0.981$, $P<0.001$),

percent of ($CD24^+/CD44^+$) cells ($r=0.983$, $P<0.001$), and cell proliferation ability ($r=0.960$, $P<0.001$).

The percent of $CD24^+$ cells was also significantly positively correlated with each of percent of $CD44^+$ cells ($r=0.998$, $P<0.001$), percent of ($CD24^+/CD44^+$) cells ($r=0.998$, $P<0.001$), and cell proliferation ability ($r=0.999$, $P<0.001$).

The percent of $CD44^+$ cells was also significantly positively correlated with each of percent of ($CD24^+/CD44^+$) cells ($r=0.998$, $P<0.001$) and cell proliferation ability ($r=0.998$, $P<0.001$).

The percent of ($CD24^+/CD44^+$) cells also showed significant positive correlation with cell proliferation ability ($r=0.998$, $P<0.001$).

Discussion

The epithelial-mesenchymal transition leads to tumor dissemination and metastatic spread. It is essential for tumor cell migration, invasion and the passing of apoptosis [23]. MTDH is an important oncogene that plays a critical role in tumor metastasis regulation [24]. However, the mechanism by which this oncogene promotes CRC aggravation is currently unknown. In the present study, we utilized MTDH up-regulation in CaCO2 cells with or without knockdown of NF-κB p65 to elucidate the molecular mechanism by which MTDH promotes CRC aggravation and identify the role of NF-κB p65 in the pathway of MTDH-induced EMT in CRC. Up-regulation of MTDH and knockdown of NF-κB p65 was confirmed by qPCR. Moreover, the ELISA technique was used to determine the protein level of NF-κB p65.

Quantitative gene expression study, herein, showed a significant increase in the gene expression of MTDH after transfection with MTDH expression plasmid in both NF-κB p65 silenced/MTDH-overexpressed CaCO2 cells (5.381fold) and MTDH-overexpressed CaCO2 cells (5.657 fold). NF-κB is a transcription factor for genes involved in cell survival, cell adhesion, inflammation, differentiation, cell growth, and tumor cell metastasis [25]. In our study, MTDH up-regulation significantly increased NF-κB p65 gene expression (about 4.205 fold) in CRC cells. In addition, the protein level of NF-κB p65 showed a significant increase in MTDH-overexpressed CaCO2 cells (5.676 ± 0.086 ng/mL) compared to cultured CaCO2 cells (2.552 ± 0.056 ng/mL). Our results were in line with Du et al. [6] who reported that MTDH activates NF-κB by promoting nuclear translocation of p65 subunit and degradation of IκBα in breast cancer.

In our study, we successfully knockdown NF-κB p65 in MTDH-overexpressed CaCO2 cells by using siRNA against human NF-κB p65. After NF-κB p65 interference in MTDH-overexpressed CaCO2 cells, the gene expression and protein level of NF-κB p65 decreased significantly and the gene expression and protein level of NF-κB p65 in silenced/MTDH-overexpressed CaCO2 cells showed no significant difference from cultured CaCO2 cells. In the present work, NF-κB p65 showed a significant positive correlation with glucose consumption, lactate production, percent of $CD24^+$, $CD44^+$, and $CD24^+/CD44^+$ cells, and cell proliferation ability.

Epithelial- mesenchymal transition is characterized by the lack of cell–cell adhesion molecules like E-cadherin. The transcription of E-cadherin is inhibited by Snail transcription factor, and increase in Snail is closely associated with EMT during cancer growth and invasion [22]. Hence, we examined the expression of Snail as a transcriptional repressor of E-cadherin. Our data demonstrated that MTDH up-regulation significantly increased the gene expression of Snail transcription factor in both NF-κB p65 silenced/MTDH-overexpressed CaCO2 cells (5.098 fold) and MTDH-overexpressed CaCO2 cells (5.252 fold).

Our result showed that MTDH gene up-regulation in CaCO2 cells significantly decreased the epithelial marker E-cadherin, while the interference with the gene expression of NF-κB p65 in MTDH-overexpressed CaCO2 cells significantly increased the gene expression of E-cadherin (3.453 fold). However, the gene expression of Snail transcription factor in NF-κB p65 silenced/MTDH-overexpressed CaCO2 cells showed no significant difference from the MTDH-overexpressed CaCO2 cells. These finding suggested that up-regulation of MTDH gene expression increased NF-κB p65 by increasing the expression of Snail transcription factor. These results identify a pathway by which MTDH regulates NF-κB p65 induced EMT during CRC cell metastasis. Our results were supported by those demonstrated by Liu et al. [26] who found that knockdown of MTDH in cervical cancer cells up-regulated the epithelial marker E-cadherin and decreased the expression of Snail transcription factor which is the key regulator of EMT.

Drug resistance is considered one of the important causes of therapy-failure in CRC. The MDR1 gene encodes the MDR1 protein which involved in multidrug resistance, as well as the elimination of xenotoxic agents [27]. Our data showed that MTDH up-regulation significantly increased the gene expression of MDR1. On the other hand, the interference with the gene expression of NF-κB p65 in MTDH-overexpressed CaCO2 cells significantly decreased the gene expression of MDR1. Our work showed that the amount of MDR1 gene expressed by MTDH-overexpressed CaCO2 cells was significantly higher than that expressed by NF-κB p65 silenced/MTDH-overexpressed CaCO2 cells (4.008 fold). Similar results were recorded by Song et al. [18] who found that MTDH induces doxorubicin resistance in the way of augmenting the expression of MDR1, leading to increased efflux and decreased the accumulation of doxorubicin.

It is widely accepted that tumor cells shift their metabolism away from respiration toward anaerobic glycolysis, to achieve excessive cell growth, proliferation, and resistance to apoptosis [20]. In the current work, glucose consumption and lactate production were measured as an indicator of the rate of anaerobic glycolysis. In this study, glucose consumption was significantly positively correlated with lactate production, percent of $CD24^+$, $CD44^+$, and $CD24^+/CD44^+$ cells, and cell proliferation ability. Lactate production was also significantly positively correlated with the percent of $CD24^+$, $CD44^+$, and $CD24^+/CD44^+$ cells and cell proliferation ability. Our results demonstrated that MTDH gene up-regulation in CaCO2 cells significantly increased glucose consumption and lactate production ability. On the other hand, the interference with the gene expression of NF-κB p65 in MTDH-overexpressed CaCO2 cells significantly decreased MTDH-induced glucose consumption and lactate production. These results were in agreement with Song et al. [20] who found that interfering with MTDH gene expression decrease the glucose consumption and lactate production in HCT116 colorectal carcinoma cells.

The proliferation of malignant tumor is a consequence of the acquisition of unlimited growth and resistance to apoptosis. It is demonstrated that MTDH potentiates serum-independent cell proliferation by inhibiting serum starvation-induced apoptosis [11]. In the current work, MTDH up-regulation significantly increased CaCO2 cell proliferation (174.903%) as assessed by MTT proliferation assay test, while interference with the gene expression of NF-κB p65 in MTDH-overexpressed CaCO2 cells significantly decreased MTDH-induced cell proliferation (97.471%). These results were online with Huang et al. [17] who find that up-regulation of MTDH enhances cell proliferation and colony formation in human CRC cells while knockdown of MTDH accumulates G0/G1-phase cells and enhances apoptosis in CRC cells. In addition, our results were in agreement with Wang et al. [28] who reported that interfering with MTDH gene expression inhibited CRC cell proliferation and invasion.

Cancer stem cell population is a subset of cancer cells responsible for invasion, metastasis, and treatment resistance in cancer. The cell surface marker CD44, CD24, and CD133 are proposed as hallmarks for colon cancer stem cells, connected with poor prognosis and aggressive cancer types [21]. In this study, we measure cell surface markers CD44 and CD24 to detect and quantify CRC stem cells. In current work, the percent of $CD24^+$ cells was significantly positively correlated with each of percent of $CD44^+$ and $CD24^+/CD44^+$ cells and cell proliferation ability. The percent of $CD44^+$ cells was also significantly positively correlated with each of percent of $CD24^+/CD44^+$ cells and cell proliferation ability. Moreover, the percent of $CD24^+/CD44^+$ cells also showed significant positive correlation with cell proliferation ability. The flow cytometric analysis, herein, showed that MTDH gene up-regulation in CaCO2 significantly increased the percent of cells acquired CRC stem cell markers ($CD24^+$, $CD44^+$, and $CD24^+/CD44^+$), while interfering with NF-κB p65 gene expression significantly decreased the acquisition of these markers. This is online with previous reports that MTDH overexpression increased cancer stem cells properties in prostate and breast cancer cells [29].

Conclusion

MTDH up-regulation could promote EMT (increase gene expression of Snail transcription factor and decrease gene expression of E-cadherin) in CRC cells and is mediated via NF-κB p65. NF-κB p65 silencing reversed the action of MTDH up-regulation on CRC aggravation. These findings at least partially reveal the molecular mechanisms by which MTDH promotes EMT of CRC and may pave the way for the development of new molecular strategies for management of CRC.

Compliance with ethical standards

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

Informed consent This article does not contain any studies with human participants or animals performed by any of the authors.

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