Research Paper

MEG3 Activated by Vitamin D Inhibits Colorectal Cancer Cells Proliferation and Migration *via* Regulating Clusterin

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ARTICLE INFO

ABSTRACT

The long non-coding RNA maternally expressed gene 3 (*MEG3*) is frequently dysregulated in human cancers; however, its roles in colorectal cancer (CRC) development are largely unknown. Here, we reported that *MEG3* was down-regulated in CRC tissues and CRC patients with lower *MEG3* showed poorer overall survival and disease-free survival than those with higher *MEG3* level. *MEG3* over-expression represses CRC cells proliferation and migration *in vivo* and *in vitro*, while *MEG3* knockdown leads to the enhanced proliferation and metastasis of CRC cells. In CRC cells, *MEG3* over-expression is related to decreased *Clusterin* mRNA and the corresponding protein levels, and it also directly binds to Clusterin protein through its 732–1174 region. In further, Clusterin over-expression rescues the compromised abilities of proliferation and metastasis induced by MEG3 over-expression, suggesting that MEG3 inhibits the CRC progression through regulating the Clusterin activities. Additionally, we found that 1α,25-(OH)$_2$D and vitamin D receptor (VDR) stimulate *MEG3* expression in CRC cells through directly binding to its promoter. These results suggested that *MEG3* functions as a tumor suppressor in CRC *via* regulating the Clusterin activities and may underlie the anticancer activities of vitamin D on CRC cells. The VDR/*MEG3*/Clusterin signaling pathway may serve as potential therapeutic targets and prognosis biomarkers for CRC patients in future.

1. Introduction

Colorectal cancer (CRC) is one of the most common malignancies of the digestive system worldwide with the incidence has being increased significantly over the past three decades (Arnold et al., 2017). >700,000 deaths has been estimated to be caused by CRC in 2012, ranking it as the 4th leading cause of cancer-related deaths (Torre et al., 2015). Although much progress has been made for CRC patients for the past decades, such as the advanced chemotherapy methods and targeted therapy including cetuximab or panitumumab, the prognosis of CRC patients is still poor. The 5-year cause specific survival of CRC patients ranges from 90.9% for AJCC 6th TNM stage I to 12.2% for those with stage IV (Lin et al., 2015). Thus, uncovering the underlying mechanisms involved in CRC development and progression may provide potential therapy targets, which may further improve the prognosis of the patients.

The development of colorectal cancer is a multi-stage and multi-step process, involving multiple genomic and epigenetic alterations (Guinney et al., 2015). Clinical studies have reported that mutation in oncogenes or tumor suppressor genes, including APC, KRAS, SMAD4 and TP53 are frequently observed in CRC patients (Yu et al., 2015). Guinney et al. have categorized CRC into four consensus molecular subtypes with distinguishing features and prognoses based on the gene expression pattern and genomic alterations of CRC tissues (Guinney et al., 2015). Recently, increasing evidence has shown that long non-coding RNAs (lncRNAs), the novel regulators in cellular signaling, play vital roles in CRC tumorigenesis and progression (Ohtsuka et al., 2016; Ma et al., 2016). lncRNA is a type of RNA transcripts longer than 200 nucleotides but do not translated into protein in cells. lncRNAs are involved in diverse cel1ular processes including cell proliferation, migration, invasion and transformation *etc.*, and dysregulation of lncRNAs are associated with various types of diseases (Ponting et al., 2009). lncRNAs regulate the gene functions through multiple levels including the chromatin modification, transcription, post-transcription, interaction with RNA-binding proteins, co-activation of transcription factors and repressors (Marchese et al., 2017). Maternally expressed gene 3 (*MEG3*),

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located on 14q32.3 of the human genome, encodes a 1.6 kilobase (kb) lncRNA (Zhou et al., 2012). It is expressed in multiple organs, including the brain, stomach, liver, pancreas and ovary. By contrast, *MEG3* expression is frequently repressed in tumor tissues (Zhou et al., 2012). In various types of cancer, genomic deletion and abnormal methylation in the promoter of *MEG3* were noticed, leading to the down-regulation of *MEG3* in tumor tissues (Bando et al., 1999; Yin et al., 2015; Lu et al., 2013). In non-small cell lung cancer (NSCLC) cells, *MEG3* inhibits proliferation and induces cell apoptosis through activating p53 and its downstream signaling pathway (Lu et al., 2013). Interestingly, for tumor cells with p53 deletion, *MEG3* over-expression also inhibits tumorigenesis through targeting the microRNAs such as microRNA-421 and microRNA-184 (Zhang et al., 2017; Li et al., 2018). These studies indicated multiple mechanisms underlying the roles of *MEG3* in tumor development. Previous studies have reported that a lower *MEG3* level was associated with the increased liver metastasis of CRC patients (Kong et al., 2016), and an enhanced CRC cells chemosensitivity to oxaliplatin (Li et al., 2017). However, the underlying mechanisms regarding the tumor suppressor activities of *MEG3* are still largely unknown. In the current study, we evaluated the anticancer activities and the underlying mechanisms of *MEG3* in CRC development and progression, which may provide potential novel intervention methods for CRC in the future.

2. Materials and Methods

2.1. Tissue Microarray Construction

Tumor specimens used in tissue microarrays (TMAs) were obtained from 371 colorectal cancer patients who underwent curative resection at Changhai Hospital of the Second Military Medical University from January 2001 to December 2010. Patients were selected with the following inclusion and exclusion criteria: (i) pathological confirmed as the primary CRC according to the World Health Organization criteria; (ii) with available formalin-fixed, paraffin-embedded (FFPE) CRC tissue samples; (iii) without any pre-operative anti-cancer treatment and no evidence of distant metastases; (iv) with complete clinicopathologic and follow-up data for the patients. Each participant provided the written informed consent and the study was approved by the Changhai Hospital Ethics Committee. The overall survival (OS) time was defined as the length of time between the surgery date and deaths by any causes. For surviving patients, the data were censored at the last following-up. The disease-free survival (DFS) was defined as the length of time between the date of the surgery and the date of tumor recurrence, metastasis or death. The tissue microarrays (TMAs) were constructed with the FFPE tissues by Shanghai Biochip Co, Ltd., Shanghai, China, following the routine protocols (Cai et al., 2017). For each patient, a 0.75-mm diameter core of the FFPE tumor tissue was punched and arranged in the TMA blocks.

2.2. Immunohistological Chemistry Staining and the in situ Hybridization

Six-micrometer thick TMA sections were used to perform immunohistochemistry staining and *in situ* hybridization (ISH) following standardized protocols (Pan et al., 2015; Deng et al., 2013). The antibody used for immunohistochemical staining of VDR was purchased from Cell Signaling Technology (Cat# 12550, RRID: AB_2637002). The lncRNA-*MEG3* probes were designed and produced by Exiqon (Vedbaek, Denmark). ISH was performed following the manufacturer's guidelines. The immunohistochemical score for each TMA sample was assessed independently by 2 pathologists.

2.3. Cell Culture

The human colorectal cancer cell lines RKO, SW1116, HT29, HCT116, LoVo, SW620, SW480 and 293 T were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. All cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin), in a humidified atmosphere of 5% CO_2 at 37 °C. Cell lines were authenticated by short tandem repeat polymerase chain reaction (STR-PCR). Mycoplasma infection status was tested by 4′, 6-diamidino-2-phenylindole (DAPI) staining in the laboratory. All colorectal cancer cell lines were used to investigate MEG3 expression, while RKO, SW1116, and LoVo were used to investigate the biological functions of MEG3. The SW1116 cell line was used to investigate the effects of MEG3 on CLU expression.

2.4. Cellular Proliferation Assay

Cellular proliferation was measured using the Cell Counting Kit-8 (CCK-8, Dojindo, Japan) kit. Cells with modified *MEG3* and Clusterin expression or not were seeded at a density of 5×10^3 cells/well in 96-well culture plates and cultured for 24, 48, or 72 h. The cells were then incubated with 10 μL CCK8 for another 4 h at 37 °C. After incubation, the viability of cells was measured at 450 nm using a microplate reader (BioTek, USA), and all experiments were repeated three times. Down-regulation of *MEG3* or Clusterin (CLU) was performed by small interfering RNA (siRNA) transfection (*MEG3* siRNA, UUAGGUAAGAGGGACA GCUGGCUGG; si-CLU1, CCAGACGGUCUCAGACAAU; si-CLU2, GGUUGA CCAGGAAAUACAA; si-CLU3, CCAGGAAGAACCCUAAAUU). Over-expression of *MEG3* or Clusterin was performed by infection of lentiviruses expressing *MEG3* or Clusterin coding regions, which were obtained from Shanghai Obio technology Company (Shanghai, China).

2.5. Tran-swell and Invasion Assays

5×10^4 cells in 200 μL serum-free Dulbecco's Modified Eagle Medium (DMEM) medium were placed in upper surface of the Transwell chambers (8 μm, Corning Costar Co., MA, USA) with the pre-coated 1:10 diluted Matrigel (BD Biosciences, CA, USA) for invasion assay or without the Matrigel for migration assay. The lower chamber was filled with 500 μL complete RPMI 1640 medium with 10% FBS. The cells were incubated for 12 to 18 h at 37 °C and then the cells on the top surface of the membrane were removed by wiping with a cotton swab. The cells that had migrated or invaded from the upper surface to the bottom surface of the filter membrane were stained with 0.5% crystal violet solution and photographed in five representative fields per insert. The cell number per field was counted and compared between the groups.

2.6. Xenograft and Metastasis Animal Models

Male BALB/c nude mice aged 4–6 weeks were obtained from Shanghai Laboratory Animal Center of China. For the tumor growth model, 1 $\times$ 10^6 SW1116 cells with or without *MEG3* stable over-expression were injected into the axillary subcutaneous tissues of nude mice. Tumor growth was determined by measuring the tumor volume, V = tumor length $\times$ tumor width^2/2 every 4 days using calipers.

For the tail vein metastasis models, 1×10^6 cells were suspended in 200 μL serum-free RPMI1640 were injected into the tail vein of nude mice. After 7 weeks, all of mice were sacrificed and the lung tissues were dissected and fixed with 10% formalin for at least 72 h. The metastasis nodules in lung tissues were analyzed by hematoxylin and eosin (HE) staining methods. All animal experiments were performed according to the guidelines on the care and use of animals for scientific use and approved by the Institutional Animal Care and Use Committee at Second Military Medical University.

2.7. RNA-pull Down Assay

RNA-pull down assay was performed as previously reported (Yuan et al., 2017). *MEG3* was *in vitro*-transcribed from the vector pSPT19-