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Knockdown of long non-coding RNA MALAT1 increases the blood–tumor barrier permeability by up-regulating miR-140

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ABSTRACT

The blood–tumor barrier (BTB) forms a major obstacle in brain tumor therapy by preventing the delivery of sufficient quantities of therapeutic drugs. Long non-coding RNAs (lncRNAs) play important roles in both normal development and diseases including cancer. Here, we elucidated the expression of lncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1) and defined its functional role in the regulation of BTB function as well as its possible molecular mechanisms. Our results proved that MALAT1 expression was up-regulated in brain microvessels of human glioma and glioma endothelial cells (GECs) which were obtained by co-culturing endothelial cells with glioma cells. Functionally, knockdown of MALAT1 resulted in an impairment and increased the permeability of BTB as well as decreased the expression of ZO-1, occludin and claudin-5 in GECs. Further, there was reciprocal repression between MALAT1 and miR-140, and miR-140 mediated the effects that MALAT1 knockdown exerted. Mechanistic investigations defined that nuclear factor YA (NFYA), a CCAAT box-binding transcription factor, was a direct and functional downstream target of miR-140, which was involved in the MALAT1 knockdown induced regulation of BTB function. Furthermore, NFYA could up-regulate the promoter activities and bind to the promoters of ZO-1, occludin and claudin-5 in GECs. Taken together, we have demonstrated the fact that knockdown of MALAT1 resulted in the increased permeability of BTB, which might contribute to establishing potential therapeutic strategies for human gliomas.

1. Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive primary human brain tumor [1,2]. Despite the benefits of surgical resection and the use of adjuvant radiotherapy and chemotherapy, the prognosis for GBM patients remains extremely poor [3]. This grim prognosis for GBM is at least partly due to the existence of blood–tumor barrier (BTB) that impairs the delivery of therapeutic drugs, resulting in the majority of drugs failure to reach the brain or do with negligible bioavailability [4,5]. Given the ineffectiveness of current treatment options, there is a critical need to improve the chemotherapy by developing successful methods to safely open BTB.

The blood–brain barrier (BBB) acts as a diffusion barrier to prevent the inflow of most compounds from blood to the central nervous system (CNS) and thereby maintains the CNS homeostasis [6,7]. BTB, similar to BBB, is characterized by the presence of tight junctions between brain capillary endothelial cells that comprises a barrier restricting paracellular diffusion [8,9]. Tight junctions are composed of transmembrane proteins (occludin and claudins) involved in intercellular contacts and cytoplasmic scaffolding proteins (zonula occludens (ZO) proteins) assembled in a multi-protein complex [8].

The human genome project revealed that a large proportion (>90%) of the human genome is actively transcribed, and the majority of transcripts are referred to as non-coding RNAs (ncRNAs) [10,11]. Based on size, non-coding RNAs are divided into small non-coding RNAs and long non-coding RNAs (lncRNAs) greater than 200 nucleotides in length [12]. The mechanisms of gene regulation by lncRNAs are much more complex and involve both activation and inhibition of gene expression [13,14]. Recent studies have shown that lncRNAs play important roles in both normal development and diseases including cancer, and participate in several

Abbreviations: GBM, glioblastoma multiforme; BTB, blood–tumor barrier; BBB, blood–brain barrier; CNS, central nervous system; ZO, zonula occludens; ncRNAs, non-coding RNAs; lncRNAs, long non-coding RNAs; MALAT1, metastasis associated lung adenocarcinoma transcript 1; miRNAs, microRNAs; NFY, nuclear factor Y; NFYA, nuclear factor YA; GECs, glioma endothelial cells; NBTs, normal brain tissues; LCM, laser capture microdissection; ECs, human cerebral microvascular endothelial cell line; HEK, human embryonic kidney; qRT-PCR, quantitative real-time PCR; TEER, transendothelial electric resistance; RIP, RNA-binding protein immunoprecipitation; ChIP, chromatin immunoprecipitation; LGG, low-grade glioma group; HGG, high-grade glioma group; ceRNAs, competing endogenous RNAs; RISC, RNA induced silencing complex.

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different biological processes [10,15]. Metastasis associated lung adenocarcinoma transcript 1 (MALAT1) is a highly conserved lncRNA in mammals, which was originally identified in metastatic non-small cell lung cancer [16]. MALAT1 has been broadly expressed in numerous tissues [17], and especially over-expressed in several human cancers including glioma [18]. Functional studies showed that its deregulation influences proliferation, invasion and/or metastasis of multiple cancer cells [19,20]. Previous study has revealed that the MALAT1 expression was increased in myocardial infarction patients and may be useful for prognostication of outcome [21]. In addition, MALAT1 up-regulation represents a critical pathogenic mechanism for diabetes-induced microvascular dysfunction [22]. However, knowledge of the MALAT1 role in regulating BTB has remained completely unknown.

In contrast to lncRNAs, small non-coding RNAs, such as microRNAs (miRNAs), have been extensively investigated both in gene regulation and cell function [23]. Expression profiling analysis has revealed a possible tumor suppressive role for miR-140 in many cancers [24–26]. Previous study also showed that miR-140 expression was significantly lower in glioma tumors as compared to non-neoplastic brain tissues [27]. Moreover, evaluation of circulating miRNAs in wet age-related macular degeneration showed that miR-140 expression was significantly down-regulated in the patients' plasma [28], and miR-140 that responded to an induced hypoxic insult was down-regulated in pericytes [29], which suggested that miR-140 might be involved in the regulation of endothelial cell function. Although miRNAs have shown to play important roles in the regulation of BTB function [30–32], the expression and function of miR-140 in BTB has not yet been reported.

Nuclear factor Y (NFY) is a CCAAT box-binding transcription factor composed of three different subunits: YA, YB and YC. Nuclear factor YA (NFYA) is the regulatory subunit and responsible for sequence-specific DNA binding [33,34]. NFYA was up-regulated in human epithelial ovarian cancer compared with normal human ovarian surface epithelial cells, and a high level of NFYA predicted poor overall survival in patients [35]. In addition, NFYA could activate multiple regulatory genes and promote self-renewal of hematopoietic stem cell [36]. However, the function of NFYA in regulating BTB has not been documented.

In this study, we sought to determine the expression of MALAT1, miR-140 and NFYA in brain microvessels of human glioma and glioma endothelial cells (GECs), and define its functional role in regulating BTB. Furthermore, the relationship among them in the regulation of BTB permeability and the possible molecular mechanisms were also investigated.

2. Materials and methods

2.1. Patients and samples

Human glioma samples and normal brain tissues (NBTs) were obtained from patients undergoing surgical resection at the Department of Neurosurgery, Shengjing Hospital of China Medical University from January 2014 to December 2014. All the patients provided written informed consent, and this project was approved by the Institutional Review Board of Shengjing Hospital of China Medical University. After resection, the tissue samples were immediately snap-frozen in liquid nitrogen. Grading of tumors was established and verified by two neuropathologists according to the World Health Organization (WHO) classification. Glioma samples were divided into two groups: low-grade glioma group (LGG, WHO I–II, $n = 8$) and high-grade glioma group (HGG, WHO III–IV, $n = 8$).

2.2. Laser capture microdissection (LCM)

LCM was used to collect enriched brain endothelium RNA as previously described [37]. Tissue samples were frozen-sectioned at 8 μm thickness via a cryostat (MICROM International GmbH, Walldorf, Germany). Sections for microdissection were stained with a fluorescent dye-tagged lectin, Ulex europaeus lectin I (UEA-I) (Vector Laboratories, Burlington, ON, Canada) according to the manufacturer's protocol. LCM was performed using the Arcturus$^{XT™}$ Microdissection Instrument. The captured microvessels were placed on CapSure® LCM Caps (Applied Biosystems, Foster City, CA, USA) and further processed for RNA isolation.

2.3. Cell lines and cultures

The immortalized human cerebral microvascular endothelial cell line (hCMEC/D3, ECs) was kindly provided by Dr. Pierre-Olivier Couraud (Institut Cochin, Paris, France). Cells were cultured on Cultrex Rat Collagen I (R&D Systems, Minneapolis, MN, USA) coated culture inserts (0.4 μm pore size; Corning, Lowell, MA, USA) in endothelial basal medium (EBM-2, Lonza, Walkersville, MD, USA) supplemented with 5% fetal bovine serum "Gold" (FBS, PAA Laboratories GmbH, Pasching, Austria), 1% Penicillin–Streptomycin (Life Technologies Corporation, Paisley, UK), 1.4 μM hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA), 1% chemically defined lipid concentrate (Life Technologies Corporation, Paisley, UK), 5 μg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA), 10 mM HEPES (PAA Laboratories GmbH, Pasching, Austria) and 1 ng/ml human basic fibroblast growth factor (bFGF, Sigma-Aldrich, St. Louis, MO, USA). The passage number was kept below 35. Human glioblastoma U87 cell line and human embryonic kidney (HEK) 293 T cell line were obtained from the Shanghai Institutes for Biological Sciences Cell Resource Center. Both U87 and HEK 293 T cell lines were cultured in high-glucose DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies Corporation, Paisley, UK). Cells were maintained at 37 °C in a humidified incubator with 5% CO_2.

2.4. Establishment of an in vitro BTB model

The in vitro BTB model was established by co-culturing ECs and U87 cells in a well-established transwell system as described previously [30,38]. Briefly, U87 cells were seeded at 2×10^4 per well in 6-well plates and cultured for 2 days prior to the addition of EC inserts. ECs were subsequently seeded at 2×10^5 per well on the apical side of inserts freshly coated with Cultrex Rat Collagen I. The co-culture was incubated for 4 days with medium replacement every 2 days. Then, the glioma endothelial cells (GECs), endothelial cells co-cultured with glioma cells, were obtained.

2.5. Reverse transcription and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Life Technologies Corporation, Carlsbad, CA, USA). High Capacity cDNA Reverse Transcription Kit (or TaqMan MicroRNA Reverse Transcription kit) was used for lncRNA and mRNA (or miRNA) reverse transcription (Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR was conducted using TaqMan Universal Master Mix II with TaqMan non-coding RNA assays of MALAT1 and GAPDH, or with TaqMan microRNA assays of miR-140 and U6, or with TaqMan gene expression assays of NFYA, ZO-1, occludin, claudin-5 and GAPDH (Applied Biosystems, Foster City, CA, USA). GAPDH and U6 were used as endogenous controls. Fold changes were calculated using relative quantification ($2^{-\Delta\Delta Ct}$) method.

2.6. Transfection and generation of stable transfected cells

Short-hairpin RNA directed against human MALAT1 was constructed in pGPU6/GFP/Neo vector (shMALAT1) (GenePharma, Shanghai,