Down-regulation of mediator complex subunit 19 (Med19) induces apoptosis in human laryngocarcinoma HEp2 cells in an Apaf-1-dependent pathway

Yan Zhao1*, Qingfeng Meng2*, Xu Gao3, Lihua Zhang1, Lixin An1

Departments of 1Allergy, 2Ophthalmology, The First Affiliated Hospital of Harbin Medical University, Harbin 150001, Heilongjiang Province, China; 3Department of Biochemistry, Harbin Medical University, Harbin 150001, Heilongjiang Province, China. *Equal contributors.

Received February 29, 2016; Accepted December 21, 2016; Epub February 15, 2017; Published February 28, 2017

Abstract: Mediator 19 (Med19) is a component of the mediator complex which is a co-activator for DNA-binding factors that activate transcription via RNA polymerase II. Accumulating evidence has shown that Med19 plays important roles in cancer cell proliferation and tumorigenesis. The physiological mechanism by which Med19 exerts its promoting effects in laryngocarcinoma is not yet fully understood. Here, we found that the expression of Med19 was increased in laryngocarcinoma samples from patients compared to normal bone tissues. Med19 knockdown significantly induced growth inhibition and suppressed migration in the HEp2 cell lines. Med19 knockdown also induced apoptosis in HEp2 cells via activation of caspase-3, 9 and Apaf-1. In addition, The tumorigenicity of Med19 short hairpin RNA (shRNA)-expressing cells were decreased after inoculating into nude mice. Taken together, our data suggest that Med19 acts as an oncogene in laryngocarcinoma via a possible caspase modulation pathway.

Keywords: Apoptosis, laryngocarcinoma, proliferation, Med19, caspase

Introduction

Laryngocarcinoma is among the most common cancers in incidence and mortality of head and neck region [1-3]. Despite improvements in diagnostic and therapeutic modalities, it has been found that no significant improvement in laryngocarcinoma survival over the past 20 years [4-6]. Thus, identification of genes and their products involved in the molecular events leading to tumorigenesis is critical for developing effective therapeutic strategies.

Med19 (ROX3) [Ensembl: ENSG000001566-03.10] was first identified during a screen for mutants with increased expression of the hemeregulated CYC7 gene and proposed to be a transcriptional regulator because of its nuclear localization [7]. The hypothesis of Med19 (ROX3) as a transcriptional regulator was further confirmed in a gene expression microarray study in Med19 deletion strain because a broad range of genes was found to be up- or down-regulated after Med19 functional disruption. Furthermore, Med19 has been demonstrated to be a component of the Mediator complex [8] and is essential for mediator binding and its activation of RNA Pol II [9, 10]. Structural analysis showed that Med19 is involved in head-module subunits in mammalian mediator complex and plays an important role in the whole mediator stabilization. Mediator, a large multiprotein complex that is structurally conserved from yeast to man [8], is responsible for the elaborate regulation of transcription that controls cell proliferation, differentiation, and maintenance. Recently, Med19 was reported to promote the proliferation of bladder cancer, hepatocellular carcinomas, prostate cancer, gastric cancer and breast cancer cells [11-15]. However, the functional role of Med19 in laryngocarcinoma cell growth and migration has not been reported.

In the present study, human laryngocarcinoma cell lines and a nude mouse model were used to study the impacts of down-regulation of Med19 in vitro and in vivo. We also explore the potential down-stream signaling molecules that mediate the biological function of Med19. Our
Down-regulation of mediator complex subunit 19 restrains laryngocarcinoma investigation may gain more insights into the progression of laryngocarcinoma cancer and provide a new target for gene therapy for this lethal disease.

**Materials and methods**

**Cell culture**

HEp2 laryngocarcinoma cells were purchased from ATCC (ATCC, Manassas, VA, USA). The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco), 100 units/mL penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2. The antibodies against Med19, caspase-3, caspase-9, Apaf-1 and β-actin were purchased from Santa.

**Construction of vectors and transfection**

Med19 siRNA (5’-AAGGTGAAGGAGAAGCTAAGT-3’) or negative control siRNA (5’-TTCTCGAGACGTCAGT-3’) were inserted into pGC-SIL-GFP lentiviral vector, respectively. The siRNA plasmids were transfected together into HEK293T cells with lentiviral helper plasmids to generate the respective lentiviruses using Lipofectamine 2000 (Invitrogen, Grand Island, NY 14072, USA). Viral stocks were made and used to infect laryngocarcinoma cells. Cells were collected for mRNA and protein levels detection after 72 h after infection.

**Real-time RT-PCR analysis**

For real-time RT-PCR analysis, total cellular RNA was extracted with Trizol (Invitrogen). Reverse transcription was performed by SuperScript II reverse transcriptase (TaKaRa code DRR037A, Dalian, China). The Med19 and internal control β-actin gene were amplified with SYBR® Premix Ex Taq™ quantitative PCR kit (TaKara code DRR041A, Dalian, China). Primers for Med19 (forward 5’-TGACAGGGACGACGAACTC-3’; reverse 5’-CAGGTCAGGCAGAAGTTAC-3’) and β-actin (forward 5’-TTAGTTGCTTACACCTCCTGTCGTTACACCTTTC-3’; reverse 5’-GCTGACCTCCTGACGTCGTCGTCGTTACACCTTTC-3’) were synthesized by Sangon (Shanghai, China). Relative expression of Med19 mRNA was calculated using the comparative CT method.

**Western blot assay**

The proteins extracted from cell lines or tissues were lysed in 1 × SDS lysis buffer (1 M Tris-HCl pH 6.8, 2% SDS, 20% glycerol, 1 mM aprotinin, 1 mM PMSF and 10 μg/mL leupeptin). The protein samples were separated by electrophoresis in SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with Tris buffer saline (TBS) containing 5% nonfat milk and 0.1% Tween 20 overnight, the membrane was subsequently incubated with primary antibodies at room temperature for 2 h or at 4°C overnight and with secondary antibody for another 2 h, respectively. The membrane was then developed using the ECL-plus™ Western blotting system (Amersham). The β-actin was used as a loading control. The experiments were repeated three times.

**Cell proliferation assay**

For determination of cell growth, An MTT cell proliferation assay was used. Cells transfected with Med19 siRNA for 48 h were seeded in 96-well plates at a density of 4000 cells per well. After incubation for 24, 48 and 72 h, 20 μL of MTT (5 mg/mL) was added. After 4 h of incubation, 100 μL of 10% dimethyl sulfoxide (SDS) containing 0.01 N HCL was added to each well. After being incubated for 24 h at 37°C, the absorbance was measured at 570 nm with a microplate reader (Bio-rad 680, USA). Cell proliferation curves were drawn according to the absorbance.

**Wound healing assay**

To assay the invasion ability of cells, each group of cells were grown on glass covers lips overnight. A wound was carried out with a sterile pipette tip, and cells were incubated in DMEM/fetal bovine serum (10%) for 48 h. At the time point of 48 h, each group of cells were fixed and visualized in microscopy. Three independent experiments were performed.

**Determination of apoptosis by nuclear staining and flow cytometry**

To assay the apoptosis of cells, each group of cells were grown on cover slips. 4,6-diamidino-2-phenylindole (DAPI) was used to check out the nuclear staining. Briefly, Cells were stained with DAPI (1 mg/mL) for 10 min, and visualized in fluorescence microscopy. Annexin V/APC and
Down-regulation of mediator complex subunit 19 restrains laryngocarcinoma

propidium iodide (PI) apoptosis detection kit (BD, CA, USA) was used to identify the rate of apoptotic cells. Three independent experiments were performed.

**Xenograft model of tumor growth in vivo**

Eighteen male nude (BALB/c nu/nu) mice, 4-6 week old, were purchased from the Chinese Academy of Sciences and housed under pathogen-free conditions in individual ventilated cages. Sterile food and water were provided ad libitum. All animal studies were approved by the local Committee for Animal Experiments. Prior to injection, 18 nude mice were randomized to three groups: Con, shCon and shMed19. A total of $4 \times 10^6$ cells in 0.2 mL of PBS were subcutaneously injected into the right flank of the nude mice. Tumor volumes were determined by external measurements and calculated according to the equation, $V = (L \times W^2) \times 0.52$ (V = volume, L = length and W = width). Mice were sacrificed after 22 days and tumor weights were measured.

**Statistical analysis**

Statistical analyses were conducted using SPSS 15.0 software. The results expressed as mean ± SD of three independent experiments. $P < 0.05$ was considered statistically significant.

**Results**

**Med19 is up-regulated in laryngocarcinoma tissues and HEp2 cells**

Aberrant expression of Med19 was detected in laryngocarcinoma (HEp2) cells by RT-PCR and Western blot analysis (Figure 1A, 1B). Med19 was expressed in all the 10 laryngocarcinoma samples, but not expressed in the adjacent normal tissues (Figure 1C).

**ShRNA targeting Med19 suppresses Med19 expression in HEp2 cells**

The silencing effects of Med19 specific shRNAs in HEp2 cells were evaluated using Western blot analysis which confirmed the down-regulation of Med19 protein by transfection of shRNA expressing lentiviruses (Figure 1D). Then, the HEp2/shMed19 cell was chosen for further experiments.

**Effect of Med19 knockdown on cell migration, growth and apoptosis**

The proliferation of Con, shCn and shMed19 cells were checked using MTT assays. Compared to shCn group, proliferation of shMed19 cells was reduced in a time-dependent manner (Figure 2A) ($P < 0.05$). No significant difference was found in proliferation between shCn and control group ($P > 0.05$).

The effect of Med19 on migration was examined by wounded healing assay. We observed that migration of shMed19 cells be significantly decreased compared to shCn cells ($P < 0.05$) (Figure 2B). Therefore, the wounded healing data showed that Med19 may play a key role in the migration of HEp2 cells.

To measure the effect of Med19 down-regulation on the apoptosis of HEp2 cells, flow cytometric analysis was performed at 48 h post-transfection. DAPI and Annexin V-APC/PI double staining were carried out to reveal the frequency of apoptosis in HEp2 cells. Cells transfected with the ShMed19, but not the control vector, showed nuclei shrinkage, and fragmented.

**Figure 1.** Detection of Med19 expression in HEp2 cell lines and tissues. A. Aberrant expression of Med19 mRNA was detected in HEp2 cells by RT-PCR. B. Aberrant expression of Med19 protein was detected in HEp2 cells by western blot analysis. C. Aberrant Med19 protein was expressed in laryngocarcinoma patient samples, but not in adjacent normal tissue. D. Med19 expression was detected by western blot after silencing of shMed19 in HEp2 cells. Con, non-transfected control cells; shNC, cells transfected with a control vector; shRNA, cells transfected with Med19-specific shRNA; Nor: normal tissue; Pat: laryngocarcinoma patient samples.
Down-regulation of mediator complex subunit 19 restrains laryngocarcinoma

The flow cytometric analysis was performed and revealed that cells treated with Med19/shRNA displayed much higher rates of apoptosis than control cells (Figure 2D). These results strongly indicate that down-regulation of Med19 can induce apoptosis in HEp2 cells.

**Med19 silencing activated Apaf-1 activity and up-regulates caspase-3, -9 in HEp2 cells**

The effects of Med19 on Apaf-1 activation in HEp2 cells were investigated. Down-regulation of Med19 expression in HEp2 cells by shMed19 effectively activated Apaf-1 levels (Figure 3A). Additionally, the apoptosis-related protein of caspase-3, -9 was increased significantly. These results suggest that Med19 can indeed promote Apaf-1 activation and may act to control HEp2 cell proliferation by regulating anti-apoptotic pathways.

**Med19 knockdown decreased the growth of laryngocarcinoma xenograft tumours in nude mice**

To further explore the tumor inhibited ability of Med19 shRNA, we used a xenograft model to examine whether Med19 shRNA inhibited tumor growth in vivo. When inoculated subcutaneously into athymus nude mice, cells treated with Med19 shRNA had dramatically reduced tumor volumes (Figure 3B) and tumor weights (Figure 3C) compared with blank control cells (Control) and control negative shRNA treated with cells, indicating that Med19 promotes the tumorigenesis of cancer cells.
Down-regulation of mediator complex subunit 19 restrains laryngocarcinoma

Discussion

Emerging evidence has demonstrated that Med19 is a novel proliferation regulator that promotes cancer cells growth and tumorigenesis, including cancers of breast, bladder, colorectal and lung [11-14]. The pathologic importance of this molecule in laryngocarcinoma cancer is yet unknown. An in-depth understanding of the molecular mechanisms underlying laryngocarcinoma proliferation is critical for the development of optimal therapeutic modalities. As a critical subunit of the Mediator complex, Med19 plays an important role in structurally stabilizing the entire Mediator complex, making it critical to the elaboration of transcriptional regulation [15]. The results from several studies have indicated that Med19 is overexpressed and plays a role in accelerating tumour growth in various cancer types. Laryngocarcinoma is characterised by an extremely high incidence of distant metastasis, particularly lung metastasis, which often correlates closely with the migration ability of tumour cells [16].

The present data demonstrated that elevated Med19 expression levels in human laryngocarcinoma tissues in comparison with normal tissue. We constructed a lentiviral system expressing Med-19-specific shRNA, and found that knockdown of Med19 in HEp2 cells remarkably reduced cell migration and proliferation ability. Activation of caspase-3 is a key and common event of two major apoptosis signaling pathways, mitochondrial pathway and death receptor pathway. The death receptor pathway activates initiator caspase-8 and then cleaves its downstream effector caspases, such as caspase-9 which transduces the apoptosis signal, while the mitochondrial pathway is initiated by the mitochondrial dysfunction and then causes activation of caspase-9 which triggers caspase-3 activation [17]. The data presented here provide the first evidence that Med19 depletion significantly increased the number of apoptotic cells in HEp2 cells which was further confirmed by the alteration of caspase-3, 9 expression, two key apoptosis-related factors [18]. Apoptotic protease activating factor 1, also known as Apaf-1, is a human homolog of C. elegans CED-4 gene. This gene encodes a cytoplasmic protein that forms one of the central hubs in the apoptosis regulatory network [19, 20]. This protein contains (from the N terminal) a caspase recruitment domain (CARD), an ATPase domain (NB-ARC), few short helical domains and then several copies of the WD40 repeat domain. Upon binding cytochrome c and dATP, this protein forms an oligomeric apoptosome [21-24]. The apoptosome binds and cleaves-caspase 9 protein, releasing its mature, activated form. Activated caspase 9 stimulates the subsequent caspase cascade that commits the cell to apoptosis. In this study, we disclosed that suppression of Med19 in HEp2 cells significantly up-regulation in Apaf-1
levels. In agreement with the in vitro results, knockdown of Med19 significantly impaired laryngocarcinoma growth and metastasis in vivo. Our data strongly suggest that Med19 is associated with the tumourigenicity of laryngocarcinoma and may be a candidate oncogene in laryngocarcinoma.

Taken together, our results provide the evidence that lentivirus mediated Med19 down-regulation inhibits laryngocarcinoma cancer cell proliferation and tumorigenesis both in vitro and in vivo, suggesting that disruption of Med19 by lentivirus transduction may be a promising approach for laryngocarcinoma cancer therapy. We propose that Med19 may be a potentially attractive and promising target for therapeutic intervention in laryngocarcinoma.

Acknowledgements

This research is supported by grants from National Nature Science Foundation of China (81400392); China Postdoctoral Science Foundation funded project (2012MS520768); Heilongjiang Postdoctoral Foundation (LBH-Z12183); The First Affiliated Hospital of Harbin Medical University Fund (2013B16, 2014B20); Heilongjiang Youth Science Fund Project (QC2014CO94).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Lixin An, Department of Allergy, The First Affiliated Hospital of Harbin Medical University, Harbin 150001, Heilongjiang Province, China. E-mail: lixin_an99@163.com

References

Down-regulation of mediator complex subunit 19 restrains laryngocarcinom


