Archives of Otolaryngology and Rhinology

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Dates: **Received:** 22 May, 2015; **Accepted:** 06 July, 2015; **Published:** 09 July, 2015

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www.peertechz.com

ISSN: 2455-1759

Keywords: Nasopharyngeal cancer; Urokinase plasminogen activator (uPA); Methyl binding domain protein 2 (MDB2); siRNA; CNE2 cell line; Methylation

Research Article

Alteration of the Methylation Status of Urokinase Plasminogen Activator (uPA) is involved in Proliferation and Invasion of Nasopharyngeal Cancer Cells

Abstract

The extracellular matrix degradation is the most important step in the process of tumor cell metastasis. Urokinase-type plasminogen activator (uPA) can catalyzes the conversion of the inactive zymogen plasminogen to the active broad-spectrum plasmin, which degrades a number of matrix proteins and also activates other proteases, including some matrix metalloproteinases. In this study, we evaluated that effect of methylation status of urokinase plasminogen activator (uPA) on invasion and metastasis of nasopharyngeal cancer cells. Methylation status of urokinase plasminogen activator (uPA) was detected by Methylation-specific PCR in the nasopharyngeal carcinoma cells. Reverse transcription-PCR was used to detect the expression of uPA mRNA in the nasopharyngeal carcinoma cell. Invasive and proliferation capacity of CNE2 cell was detected by using Boyden chamber Matrigel invasion assay and Cell proliferation assay after MBD2 knockdown. Our data demonstrated that uPA gene promoter hypomethylation is related to strong uPA mRNA expression in nasopharyngeal cancer cell, uPA mRNA expression was significantly reduced after MBD2 knockdown in the CNE2 cells, the proliferation and invasion capacity of CNE2 cells was significantly inhibited after MBD2 knockdown. Our data suggest that reversal of uPA gene hypomethylation will become a novel therapeutic approach for blocking nasopharyngeal cancer progression and metastatic.

Introduction

A major feature of cancer cells is their ability to migrate and to invade and develop in surrounding or distant tissues. Tumor cells metastasis to distant organs involves four major steps [1]: adhesion of tumor cells to the extracellular matrix (ECM), ability of tumor cells to degrade the ECM and intravasate into surrounding blood vessels, survival against the natural host defenses and settling at the preferred organ site, and extravasation into the organ and formation of new tumors. The extracellular matrix degradation is the most important step in the process of tumor cell metastasis. Urokinase-type plasminogen activator (uPA) is a member of the serine protease family. It catalyzes the conversion of the inactive zymogen plasminogen to the active broad-spectrum plasmin, which degrades a number of matrix proteins and also activates other proteases, including some matrix metalloproteinases [2]. It has been implicated that uPA plays an important role in the process of invasion, metastasis and angiogenesis of several malignancies [3-5].

Nasopharyngeal cancer (NPC) is a common tumor in head and neck. It has an unusually high incidence in southern China. Cervical lymph node metastasis is a predominant clinical characteristic in the earlier period of nasopharyngeal cancer. It is not very clear that the mechanism of nasopharyngeal cancer metastasis. We have previous study showed that methylation status of Death-associated protein kinase (DAPK), a tumor suppress gene, plays an important role in nasopharyngeal carcinoma progression and metastasis [6]. Recent studies have found that the hypomethylation of oncogene plays a causal role in tumor metastasis. Pakneshan found that hypomethylation is responsible for uPA activation and plays a role in metastasis, inhibition of demethylation result in methylation and inactivation of uPA and reversal of metastasis in breast cancer [7].

In the present study, we demonstrate that methylation state and expression of uPA gene, and its hypomethylated state could be reversed pharmacologically by agents that modulate methylation like an antisense inhibitor of Methyl-binding protein 2 (MDB2) in nasopharyngeal cancer cell line CNE2. This study confirms that uPA is activated by hypomethylation in nasopharyngeal cancer cells, and uPA plays a important role in proliferation and invasion of nasopharyngeal cancer cell. It suggests that pharmacological reversal of uPA gene hypomethylation can be used as a novel therapeutic approach for blocking nasopharyngeal cancer progression.

Materials and Methods

Cell culture and treatment

Human nasopharyngeal cancer cell line CNE2 was obtained from the China Center for Type Culture Collection (CCTCC). CNE2 cell lines were maintained in RPMI 1640 with 10% Fetal bovine serum (FBS), 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate. Cells were incubated at 37°C in 5% CO2.

Plasmid construction

The targeting sequences corresponding to the siRNA for MBD2 were selected from GeneBank Accession No. NM_003927.4 (GAAGTTCAGAAGCAAGCCT). The pEGFP-C1–U6 plasmid, which had been constructed from pEGFP-C1 and U6 promoter by cloning technique, was digested with BamHI and HindIII. Then, the annealed oligos were ligated into the plasmid. The sequence is BamHI + sense + loop + antisense +termination signal + SalI + HindIII 5′-GATCC GAAGTTCAGAAGCAAGCCTTTCAAGACGAGGCTTGCTTCTGAACTTCTTTTTTGTCGACA-3′. The sequence encoding siRNA with 19 nt of homology to MBD2 was inserted to the down-stream of U6 promoter between BamHI and HindIII. The constructed plasmid was confirmed by DNA sequencing and named pEGFP-C1-U6-MBD2. The target sequence of unrelated siRNA controls (UC) was GACTTCATAAGGCGCATGC. The plasmid constructed using the unrelated siRNA controls sequence was named pEGFP-C1-U6-UC.

Cell transfection

CNE2 cells were seeded at an initial density of 104 per well in six well plates. When the cells reached 30% to 50% confluency, cells were transfected with appropriate plasmids (pEGFP-C1-U6-MBD2 or pEGFP-C1-U6-UC) by using Lipofectamine 2000 (Invitrogen, USA) in accordance with the manufacturer's protocol. At 48 hours post-transfection, 0.8mg/ml G418 (Sigma, USA) was added to select positive cell clones. Stable cell clones were isolated after 8 weeks. 104 cells without transfection, cells transfected with control vector or cells transfected with siRNA were seeded into a 6-well cell culture plate, respectively. Cells of each group were harvested after culture for 72 h. It was used for followed experiment.

Methylation-specific PCR (MSP)

Nasopharyngeal cancer cell genomic DNA was extracted using the DNeasy tissue kit and was modificated using the Zymo golden methylation kit (Zymo, Orange, CA) following the manufacturer's instructions. Methylation-specific PCR was done to amplify the indicated region of uPA gene. The methylated primer: forward 5′-AGC GTT GCG GAA GTA CGCGG-3′and reverse 5′-AAA CCC GCC CCG ACG CCG CC-3′). The unmethylated primer: forward 5′-AGT GTT GTG GAA GTA TGT GG-3′ and reverse 5′-AAA CCC ACC CCA ACACCA CC-3′). The above uPA promoter using Platinum Taq DNA polymerase (Invitrogen) according to the manufacturer's recommendations under the following same cycle conditions: 95℃ for 3 min; 10 cycles of 95℃ for 30 s, 52℃ for 30 s and 72℃ for 45 s; 20 cycles of 95℃ for 30 s, 50℃ for 30 s and 72℃ for 45 s; a final extension of 72℃ for 5 min. The amplified products were fractionated on 2% agarose gels.

Reverse transcription-PCR (PT-PCR)

Total RNA was isolated from nasopharyngeal cancer cells using Trizol regents. It was used for reverse transcription and amplification. The following primers were used for RT-PCR. The uPA primer: 5-ACA TTC ACT GGT GCA ACT GC-3 (forward) and 5-TTT CCC CAG GCC CAG CTG AA-3 (reverse). The MBD2 primer: 5-AGC GAT GTC TAC TAC TTC AG-3 (forward) and 5-AGA TGT CTG CCA TCA GTG CT-3 (reverse). The β-actin primer: 5′-CGT CTG GAC CTG GCT GGC CGG GACC-3′ (forward) and 5′-CTA GAA GCA TTT GCG GTG GAC GAT G-3′ (reverse). The DNA was amplified under the following conditions: 95°C for 3 min, 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR products were then analyzed on a 1% agarose gel.

Boyden chamber Matrigel invasion assay

We examined the invasive capacity of control and experimental CNE2 cell using two compartments: Boyden chambers (Transwell Costar, Cambridge, MA) and basement membrane Matrigel (BD Biosciences, Mississauga, Ontario, Canada). All cells were analyzed for their viability and an equal number of viable cells (10^4) was added to the upper chamber and allowed to invade through the Matrigel onto the filters for 18 hours. At the end of the incubation period, the filters were washed, fixed, and stained. The invading cells were then examined and counted in 10 randomly selected fields under a light microscope at×400 magnification. Ten random fields for each set of experiments were analyzed and the average number of cells invaded was calculated.

Cell proliferation assay

Three kinds of cells (cells without transfection, cells transfected with control vector and cells transfected with siRNA), were seeded into five 6-well culture plates, with each plate having all three kinds of cells and each group consisted of two parallel wells. All cells, plated at exactly the same density of the viable cells per plate in triplicates, were trypsinized and the number of viable cells was counted daily throughout the 5-day treatment. The growth curve of each group was plotted on the basis of each day cells number.

Statistical analysis

Results are expressed as the mean±SE. Statistical comparisons were performed using Student's t-test analysis of variance. A probability value of $p< 0.05$ was considered to be significant.

Results

Effect of MBD2 knockdown on status of uPA gene methylation

Methylation status of the uPA promoter was analyzed by using MSP in nasopharyngeal cancer cell line (CNE2). Hypomethylation of uPA gene promoter was found in CNE2 cells transfected with plasmids pEGFP-C1-U6-UC and without any transfection. Hypermethylation of uPA gene promoter was found in the CNE2 cells transfected with plasmids pEGFP-C1-U6-MBD2.These results showed that promoter hypomethylated uPA gene was re-methylated by transfected pEGFP-C1-U6-MBD2 in CNE2 cells (Figure 1).

Effect of MBD2 knockdown on uPA mRNA expression

To determine the effect of MBD2 knockdown on uPA mRNA expression, three kinds of cells (cells without transfection, cells transfected with pEGFP-C1-U6-MBD2 or pEGFP-C1-U6-UC plasmid) were seeded into 6-well culture plates and maintained for 72 hours. Then, total RNA of cells were extracted and isolated. The level of MBD2 mRNA was detected by using RT-PCR as described in

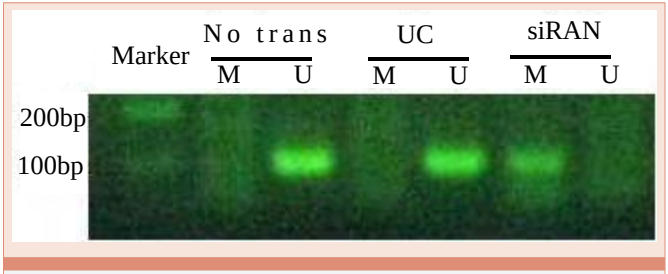

Figure 1: Effect of MBD2 knockdown on status of uPA gene methylation. No Trans, cells without any transfection; siRNA, cells transfected with pEGFP-C1-U6-MBD2; UC, cells transfected with pEGFP-C1-U6-UC plasmid M, Methylated production; U, unmethylated production.

Materials and Methods. RT-PCR analysis indicated that significant down regulation of MBD2 mRNA expression was found in the CNE2 cells transfected pEGFP-C1-U6-MBD2 plasmid than the CNE2 cells transfected pEGFP-C1-U6-UC plasmid or without any transfection (Figure 2). This result showed that MBD2 gene was silenced by transfected pEGFP-C1-U6-MBD2 plasmid.

RT-PCR was carried out to determine the resulting effect of inhibiting MBD2 mRNA expression on the expression of uPA mRNA. The results showed that uPA mRNA expression was significant reduce in CNE2 cells transfected pEGFP-C1-U6-MBD2 plasmid than the CNE2 cells transfected pEGFP-C1-U6-UC plasmid or without any transfection (Figure 3). These studies show that MBD2 gene expression regulate uPA gene expression in the CNE2 cells.

MBD2 knockdown repress invasive capacity of CNE2 cells

The invasive capacity of CNE2 cells was examined by using Boyden chamber Matrigel invasion assay. As expected, invasive cell count in the CNE2 cells (=15.60 ±1.51) transfected pEGFP-C1-U6-MBD2 plasmid was significantly decrease than the CNE2 cells (=25.40 ±2.22) transfected pEGFP-C1-U6-UC plasmid (t=10.628 ,P = 0.000) or the CNE2 cells (=25.00 ±2.36) without any transfection (t=11.549 ,P = 0.000) (Figure 4). The invasive cell count is no different between the CNE2 cells transfected pEGFP-C1-U6-UC plasmid and the CNE2 cells without any transfection. These studies show that uPA gene plays an important role in maintaining the invasive capacity of CNE2 cells.

The effect of MBD2 knockdown on CNE2 cells proliferation

The growth curve of each cell group showed that cell proliferation was slower in the cells transfected with pEGFP-C1-U6-MBD2 plasmid as compared with the cells transfected with pEGFP-C1-C6-UC plasmid or cells without any transfection (Figure 5).

Discussion

The majority of nasopharyngeal carcinoma is poorly differentiated squamous carcinoma or undifferentiated carcinoma. Cervical lymph node metastases are often early symptoms of nasopharyngeal carcinoma. Radiotherapy is the main treatment for nasopharyngeal cancer. All chemotherapy drugs of treatment nasopharyngeal carcinoma are not very ideal. In the present, the research of gene therapy is more and more attention. Changes of DNA methylation status at the CpG islands of gene promoters are the most common molecular alterations in human cancers [8]. In recent years, it has become increasingly obvious that DNA hypermethylation of tumor suppressor gene promoter play an important role in some cancer. Many studies have demonstrated that the CpG islands of multiple cancer-related genes are frequently methylated in a variety of human cancers and expression of the corresponding gene is silenced [8-10]. DNA methylation is a reversible biochemical modification [11]. Tumor suppressor gene transcriptional inactivation caused by CpG island methylation can be reversed by DNA methyltransferase inhibitor (5 - aza-2′-deoxycytidine). The reversal (CpG island demethylation) can restore the function of tumor suppressor genes, thereby inhibiting tumor cell growth and proliferation [12]. Therefore, to restore the function of tumor suppressor genes by using DNA methyltransferase inhibitors has become one of the new means of cancer gene therapy. Our previous study demonstrated that loss of DAPK expression is associated with aberrant promoter region methylation in nasopharngeal cancer cell line (CNE2) and laryngeal cancer cell line (Hep-2), 5 - aza-2′-deoxycytidine may reactivate DAPK genes silenced by promoter region hypermethylation and slow the growth of Hep-2 cells and CNE2 cells in vitro and in vivo [6,13]. However, Growing evidence now indicates that demethylation of oncogene also plays a causal role in carcinogenesis. For example, SFN [14], hsa-miR-191 [15], Neurog1 [16], p-cadherin [17] and r-ras [18] genes are activated by DNA demethylation in various tumors. However, how did it keep the hypomethylation statue of oncogene in the cancer cells? Some scholars suppose that the demethylase might

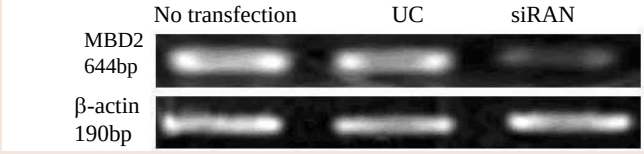

Figure 2: Expression of MBD2 mRNA in nasopharyngeal cancer cells. No transfection, cells without transfection; siRNA, cells transfected with pEGFP-C1-U6-MBD2; UC, cells transfected with pEGFP-C1-U6-UC plasmid. Transfection of pEGFP-C1-U6-MBD2 plasmid resulted in a significant reduction in the levels of MBD2 mRNA in nasopharyngeal cancer cells.

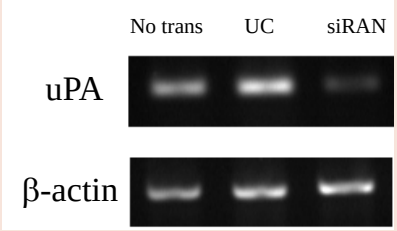

Figure 3: Effect of MBD2 knockdown on uPA mRNA expression No transfection, cells without transfection; siRNA, cells transfected with pEGFP-C1-U6-MBD2; UC, cells transfected with pEGFP-C1-U6-UC plasmid. uPA mRNA expression was significantly down-regulated in CNE2 cells transfected with pEGFP-C1-U6-MBD2 plasmid compared with CNE2 cells without any transfection or transfected pEGFP-C1-U6-UC plasmid.

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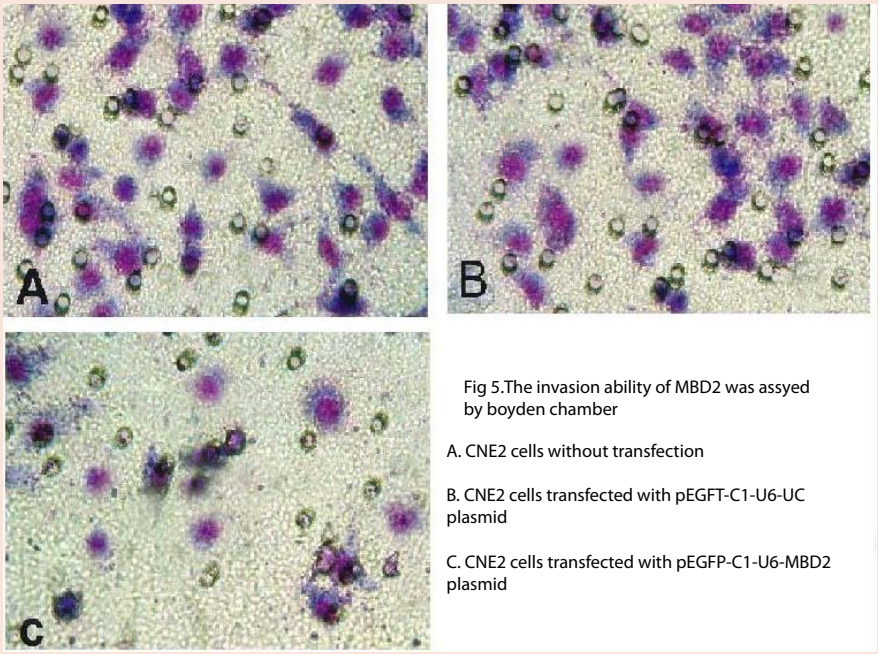

Figure 4: MBD2 knockdown repress invasive capacity of CNE2 cells.
Invasive capacity of CNE2 cells transfected with pEGFP-C1-U6-MBD2 was significantly decreased compared with CNE2 cells without any transfection or transfected pEGFP-C1-U6-UC plasmid.

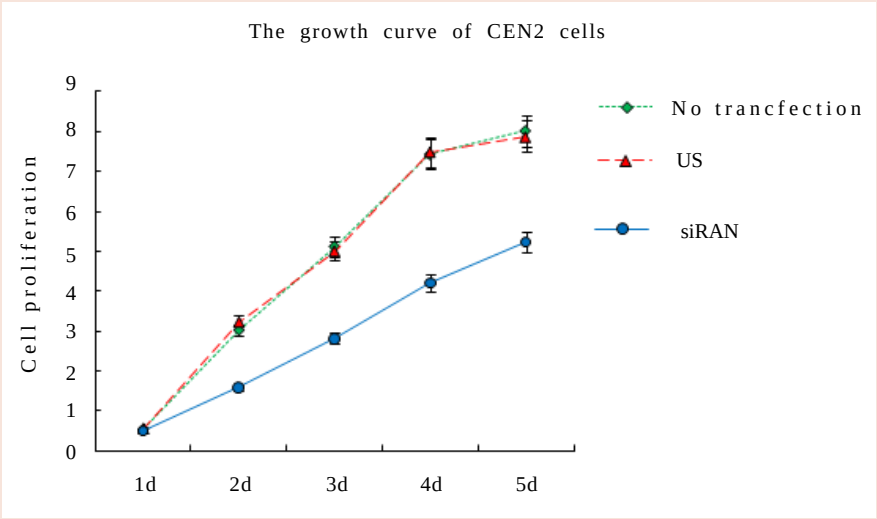

Figure 5: The effect of MBD2 knockdown on CNE2 cells proliferation.
Cell proliferation was slower in the CNE2 cells transfected with pEGFP-C1-U6-MBD2 plasmid than the CNE2 cells without any transfection or transfected pEGFP-C1-U6-UC plasmid.

play a key role in hypomethylation statue of oncogene. But, so far no one demethylase was verificated.

The inactivity of hypermethylation-associated gene is carried out by methyl-binding proteins specifically bind to methylated CpG sites. Methyl-binding proteins could inhibit transcription of the promoter hypermethylation gene, it play an important role in hypermethylation gene inactivation. Methyl-binding protein 2 (MDB2) is one of the members of the methyl-binding protein family. Pakneshan [7] found that MBD2 not only has a function of inhibit transcription of hypermethylation gene, but also can maintain certain genes hypomethylated in tumorgenesis. MBD2 knock-down may cause demethylation reaction weakened and methylation enhancements in cancer cells. It can make inactivation of hypomethylation oncogene by de novo methylation and inhibit the growth and metastasis

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in cancer cells. Pakneshan inhibited the expression of MBD2 by using antisensenucleic acids results in silencing of uPA, increased methylation of the uPA promoter, and inhibition of invasion in vitro and metastasis in vivo in breast cancer cells [7]. In the study, MBD2 gene was silenced by using RNA interference technology. It was found that promoter hypomethylated uPA gene was re-methylated, uPA mRNA expression was significantly decrease, the proliferation and invasion capacity of cells was significantly inhibited after MBD2 knockdown in the CNE2 cells. Those results showed that uPA gene expression was regulated by MBD2 gene, the status of uPA gene expression is relation to the proliferation and invasion capacity of cells in the CNE2 cells.

In short word, the mechanism which MBD2 make inactivation of uPA gene hypomethylated by de novo methylation is not very clear. But, we can regulate the expression of oncogenes or tumor suppressor genes by regulating the activity of methyltransferase. We can further regulate growth and proliferation of cancer cells. So, the study changed the methylated status of oncogenes or tumor suppressor genes will become a new research target in gene therapy of cancer.

Acknowledgement

Grants for this study came from the Research Fund for the Doctoral Program of the Ministry of Education of China (No.20070487172).

This paper is an experimental study

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