

## **Transcription of synaptic plasticity-related genes in patients with somnipathy combined with type 2 diabetes.**

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### **Abstract**

**Objective:** To investigate DNA methylation and hydroxymethylation in patients with somnipathy combined with type 2 diabetes, and to explore the effects of DNA methylation and hydroxymethylation on gene expression.

**Methods:** Thirty patients with somnipathy combined with type 2 diabetes and 20 patients with type-2 diabetes but without somnipathy were considered. DNA methylation of Disks Large Homolog 4 (*DLG4*) and Ras-related protein Rab-11 (*Rab11*) was detected by bisulfite sequencing and DNA hydroxymethylation of activity-regulated cytoskeleton-associated protein (*Arc*), Cyclic AMP-Responsive Element-Binding protein 3 (*CREB3*) and Early Growth Response protein 1 (*EGR1*) was analyzed by CHIP analysis. Transcription levels of *DLG4*, *Rab11*, *Arc*, *CREB3* and *EGR1* were detected by quantitative real-time RT-PCR (qRT-PCR).

**Results:** Methylation levels of *DLG4* and *Rab11* and hydroxymethylation levels of *Arc*, *Creb3* and *Erg1* in patients with somnipathy were significantly higher than those in control group ( $p < 0.01$ ). Increased transcription levels of *DLG4*, *Arc* and *Erg1*, and decreased transcription levels of *Rab11* and *Creb3* were found in patients with somnipathy than in patients without somnipathy. Transcription level of *DLG4* was positively, and *Rab11* was negatively correlated with their methylation levels. Transcription levels of *Arc* and *Erg1* were positively, and transcription level of *Creb3* was negatively correlated with hydroxymethylation levels.

**Conclusion:** Increased methylation levels of *DLG4* and *Rab11* and hydroxymethylation levels of *Arc*, *Creb3* and *Erg1* were related to the development of type 2 diabetes in patients with somnipathy. Methylation and hydroxymethylation can significantly affect gene expression at transcription level.

**Keywords:** Type 2 diabetes, Somnipathy, DNA methylation, Bisulfite sequencing.

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### **Introduction**

Somnipathy refers to the abnormal sleep patterns that can seriously affect normal, mental, emotional and physical functioning of patients [1]. Somnipathy can affect both children and adults [2,3]. According to 2014 report of National Sleep Foundation, the average sleeping time of children between 6-17 y is 1-2 h shorter than the recommended, resulting in emotional and physical problems. An investigation on workers has shown that, chronic sleep deprivation caused by long work hours was found to be closely correlated poor work outcomes including occupational accidents presenteeism and absenteeism [4]. Numerous clinical studies have shown that poor sleep quality and chronic sleep disorders can contribute to the onset, development and progression of metabolic diseases including insulin resistance and obesity [5-7]. Sleep disorders are usually accompanied by the emergence of endocrine disorders and inflammatory response, which may eventually lead to the occurrence of type 2 diabetes

but not type 1 [8-12]. It's well accepted that DNA methylation and hydroxymethylation are closely correlated with the prevalence of type 2 diabetes, and the control of DNA methylation may provide new opportunities for the prevention of this disease [13,14]. Sleep disorder can also be significantly affected by epigenetic factors. It has been proved that that epigenetic factors could regulate the expression of genes involved in circadian clock networks at both transcriptional and post-transcriptional levels [15]. As an important component of circadian clock system, sleep plays pivotal roles in regulating fundamental biological rhythms controlled by circadian clock. In addition, sleep disorder can regulate gene expression in mouse brain through DNA methylation and hydroxymethylation to affect stress response and circadian rhythm [16]. In view of the pathogenesis of type 2 diabetes and the effects of sleep disorder on DNA methylation and hydroxymethylation, it will be reasonable to hypothesize that sleep disorder can also affect the development of type 2

diabetes by regulating DNA methylation and hydroxymethylation.

In this study, methylation and hydroxymethylation of synaptic plasticity-related genes were detected in type 2 diabetes patients with or without somniphathy. Correlations between gene transcription, methylation, and hydroxymethylation were also analyzed. The report is as follow.

## Materials and Methods

### Objects

Thirty patients with somniphathy combined with type 2 diabetes were selected in the First Affiliated Hospital of Xinjiang Medical University from January 2015-2016 according the criteria described in ICSD-3.

**Inclusion criteria:** (1) With complaints of insomnia, including difficulties in falling asleep or sleep maintenance and early morning awakenings; (2) With daytime functional impairment, including fatigue, emotional problems and lack of motivation; (3) With symptoms cannot be explained by the lack of sufficient opportunity or suitable environment; (4) With frequency of sleep disorder greater than three times per week and duration longer than three months.

**Exclusion criteria:** (1) Patients with sufficient sleeping opportunities; (2) Patients with trauma, severe liver and kidney disease, coronary heart disease, high blood pressure, blood disease, cancer and autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus etc. Among the 30 patients, there were 20 males and 10 females with an average age of  $45 \pm 8.32$  y. 20 type 2 diabetes patients without somniphathy were selected at the same time to serve as control group. Patients in control group included 11 males and 9 females with an average age of  $47 \pm 6.94$  y. No significant differences in age, gender, Body Mass Index (BMI) and other basic information were found between those two groups.

### DNA and RNA extraction

Blood was extracted from each patient. Genomic DNA and total RNA were extracted from blood samples using a blood DNA extraction kit (No. 51104, Qiagen, Hilden, Germany) and a blood RNA extraction kit (No. 51504, Qiagen, Hilden, Germany), respectively. All operations were performed in strict accordance with the instructions. DNA and RNA samples were quantified using NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

### Detection of DNA methylation

DNA methylation was detected using EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, CA, USA) according to the principle of bisulfite sequencing. After bisulfite modification, PCR reaction was performed using the modified DNA as template. PCR products were sequenced to identify methylation sites by comparing to the original sequences.

Sequences of all primers (Sangon, Shanghai, China) used in PCR reaction were listed in Table 1.

**Table 1.** Sequences of primers used in PCR reactions.

Genes	Primers	Sequences
DLG4	Forward	TGGTAGGGAATATGTGTGTTT
	Reverse	ACCTAAACTCTCCTTAAAACTCT
	Forward	AGTTTTTTTTTGGGGAGGAAAGAG
	Reverse	ACCCCTAAAATAATCCCTTTATAC
Rab11	Forward	ATCACCTCTCAGGAAAGTCTAAG
	Reverse	CTTCGAGAAATGTGCCATAATCTGAC
	Forward	ATTTAGGGATTTTAAATTAGAGATTT
	Reverse	CCTAACTCTACAATTCCAAAAAAC
Arc	Forward	GGTAAATGGGAGTTAGGGTTT
	Reverse	TCATATAATCCAACCTCCATCTACTC
	Forward	TGTGATTTTGTAGATTGGTAAGTGT
	Reverse	CAAACCTTAATAAATCTTCTCCAAC
Creb3	Forward	TGAGGGAATGCGATAAGTTGC
	Reverse	TGGTCCAGTTCTGTCTGAGTATT
	Forward	TTTCAGTGTGAACCATCCGAG
	Reverse	AGCACCTTCTTGAGAACTCAG
Erg1	Forward	GATCCGAGGAGCACAGAC
	Reverse	GTGAGCCGAGCAGAAAAAC
	Forward	GCGAAGCCCTAAGACAAT
	Reverse	CACCCAGATGAAATCCCTAC

### Detection of DNA hydroxymethylation

Genomic DNA (3 µg) was broken into small fragments using ultrasonic waves, and fragments ranged from 200 to 500 bp were harvested using Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). DNA fragments were then subjected to CHIP reaction using hMeDIP kit (Diagenode, Beijing, China) to analyze DNA hydroxymethylation.

### Quantitative real-time RT-PCR (qRT-PCR)

Oligo (dT) 15 primer (Sangon, Shanghai, China) and AMV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) were used to synthesize cDNA through reverse transcription using RNA as template. SYBR Primemix Ex Taq™ kit (Takata, Dalian, China) and the synthesized cDNA were used to prepare PCR reaction system. Following primers were used in PCR reaction: *DLG4*, qHsaCEP0051707 (Bio-Rad, Hercules, CA, USA); *Rab11*, qHsaCID0010203 (Bio-Rad, Hercules, CA, USA); *Arc*, qHsaCED0048333 (Bio-Rad, USA); *Creb3*, qHsaCID0016155 (Bio-Rad, Hercules, CA, USA); *Erg1*, qHsaCID000773 (Bio-Rad, Hercules, CA, USA); GAPDH sense primer: 5'-GAGTCAACGGATTTGGTCTG-3'

and GAPDH antisense primer: 5'-TTGATTTTGGAGGGATCTCG-3'. PCR reaction conditions were: 94°C for 30 s, followed by 40 cycles of 94°C for 5 s and 55°C for 45 s. Data were processed using 2<sup>-ΔΔCt</sup> method. Relative expression level of each gene was normalized to endogenous control GAPDH.

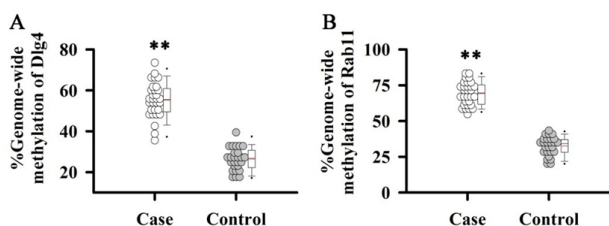
**Statistical analysis**

SPSS19.0 (SPSS Inc., Chicago, IL, USA) software was used to analyze the data. Comparisons between groups were performed by t-test. p<0.05 was considered to be statistically significant.

**Results**

**Methylation of DLG4 and Rab11**

Studies have shown that DNA methylation could significantly affect the expression of *DLG4*, *Rab11* and other genes in brain tissue of mice. Therefore, methylation levels of *DLG4* and *Rab11* in patients with somniphathy were investigated in this study. Results showed that methylation levels of *DLG4* (Figure 1A) and *Rab11* (Figure 1B) in patients with somniphathy were significantly higher than those in control group (p<0.01).

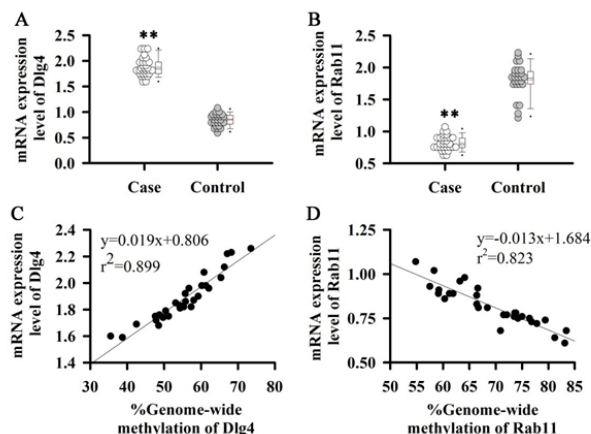


**Figure 1.** Methylation of *DLG4* and *Rab11* in patients with somniphathy. Note: \*\*Compared with patients without somniphathy, p<0.01.

**Effect of methylation on transcription of DLG4 and Rab11**

DNA methylation is a common mechanism of transcriptional regulation. Methylation of the upstream transcriptional regulation region can often lead to gene silencing, while methylations within genes affect the process of transcriptional regulation or alternative splicing. Therefore, expression levels

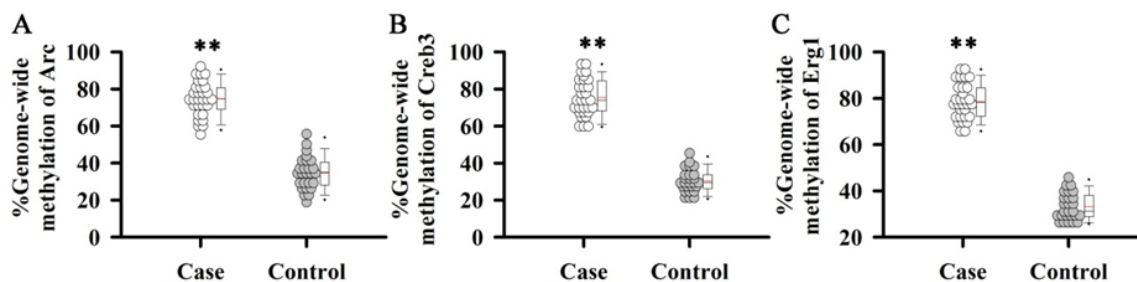
of *DLG4* and *Rab11* mRNA in serum of patients with somniphathy were detected in this study. As shown in Figure 2A, expression level of *DLG4* mRNA in patients with somniphathy was significantly higher than that in control group (p<0.01) and was positively correlated with its methylation level (Figure 2C). While expression level of *Rab11* mRNA in patients with somniphathy (Figure 2B) was significantly lower than that in control group (p<0.01) and was negatively correlated with its methylation level (Figure 2D). Those results suggested that DNA methylation could up-regulate the transcription of *DLG4* but down-regulate the transcription of *Rab11*.



**Figure 2.** Effects of methylation on transcription of *DLG4* and *Rab11*. (A) Effects of methylation on transcription of *DLG4*; (B) Effects of methylation on transcription *Rab11*; (C) Correlation between the methylation level and transcription level of *DLG4*; (D) Correlation between the methylation level and transcription level of *Rab11*. Note: \*\*Compared with patients without somniphathy, p<0.01.

**Hydroxymethylation of Arc, Creb3 and Erg1**

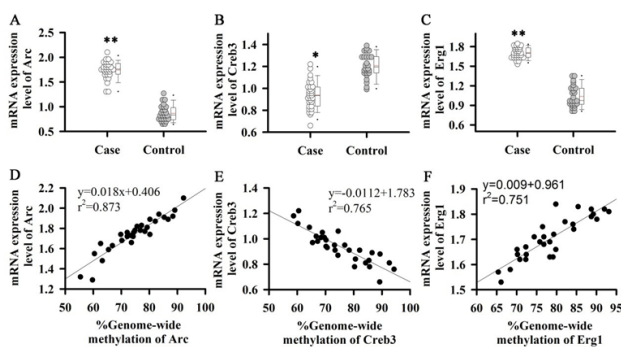
In addition to methylation, hydroxymethylation also plays an important role in the regulation of gene expression. Therefore, hydroxymethylation levels of *Arc*, *Creb3* and *Erg1* genes were detected. Results showed that the hydroxymethylation levels of *Arc* (Figure 3A), *Creb3* (Figure 3B) and *Erg1* (Figure 3C) were significantly increased in patients with somniphathy compared with patients without somniphathy (p<0.01).



**Figure 3.** Hydroxymethylation of *Arc*, *Creb3* and *Erg1* in patients with and without somniphathy. (A) Hydroxymethylation of *Arc*; (B) Hydroxymethylation of *Creb3*; (C) Hydroxymethylation of *Erg1*. Note: \*\*Compared with patients without somniphathy, p<0.01.

## Effects of hydroxymethylation on transcription of *Arc*, *Creb3* and *Erg1*

DNA hydroxymethylation plays an important role in regulating gene expression. Therefore, effects of hydroxymethylation on transcription levels of *Arc*, *Creb3* and *Erg1* were investigated in this study. Results showed that transcription levels of *Arc* and *Erg1* were significantly increased in patients with somniphathy (Figures 4A and 4C), and were positively correlated with their hydroxymethylation levels (Figures 4D and 4F). While transcription level of *Creb3* was significantly decreased in patients with somniphathy (Figure 4B), and was negatively correlated with its hydroxymethylation level (Figure 4E). Those results suggested that hydroxymethylation could up-regulate the transcription levels of *Arc* and *Erg1*, but down-regulate the transcription level of *Creb3*.



**Figure 4.** Effects of hydroxymethylation on transcription of *Arc*, *Creb3* and *Erg1*. (A) Effects of hydroxymethylation on transcription of *Arc*; (B) Effects of hydroxymethylation on transcription of *Creb3*; (C) Effects of hydroxymethylation on transcription of *Erg1*; (D) Correlation between hydroxymethylation level and transcription level of *Arc*; (E) Correlation between hydroxymethylation level and transcription level of *Creb3*; (F) Correlation between hydroxymethylation level and transcription level of *Erg1*.

## Discussion

Sleep disorder is closely related to the development of various human diseases. In the study of Parkinson disease, Friederike et al. reported that Rapid Eye Movement (REM) sleep behaviour disorder, which is associated with aging, was a frequent disturbance for all stages of PD without preferred PD subtypes and gender [17]. In another study, fatigue caused by poor sleep was proved to be one of the main causes of inflammatory bowel disease [18]. Interactions between immune system and brain were mediated by a complex network formed by cytokines, endocrine hormones and autonomic nerves, while sleep disorders can disturb the function of this network to increase the levels of inflammatory mediators to participate in the progression of cardiovascular diseases [19]. Sleep controls daily physiological patterns which is important for normal metabolic health. Sleep deficiencies caused by circadian misalignment, night eating syndrome, shift work, sleep apnea, insufficient sleep schedules, narcolepsy and many other factors can potentially cause metabolic dysregulation, leading to the occurrence of various metabolic diseases including type 2 diabetes, obesity etc., [20]. Numerous

studies have shown that the reduced sleep quality could contribute to the development of type 2 diabetes [21]. A recent large-scale clinical study has shown that patients with sleep insufficiency was more likely to develop type 2 diabetes compared with the ones with sufficient sleep and quality and quantity of sleep can be used to predict the risk of the progression of type 2 diabetes [22]. In clinical treatment of type 2 diabetes, insufficient sleep was found to be significantly related to the poor treatment outcomes of blood glucose control [23]. Besides insufficient sleep, long sleep was also found to be positively correlated with the development of various diseases including diabetes, obesity, cardiovascular diseases and hypertension, and sleep duration of 7-8 h can directly and indirectly decrease the risk of chronic disease [24].

It has been shown that methylation plays an important role in the occurrence, development, and progression of sleep disorder-mediated diseases. As a regulator of T regulatory lymphocytes, methylation level of *FOXP3* gene was found to be significantly increased in children with obstructive sleep apnea, and the increased methylation level of *FOXP3* can eventually lead to the emergence of inflammatory responses [25]. Therefore, methylation level of *FOXP3* can potentially be a biomarker for the vulnerability of organs. A recent study has shown that wakefulness in a single night is sufficient to alter the transcriptional profiles of genes involved in circadian clock system in key metabolic tissues through DNA methylation, which can be used to explain the disrupted metabolic integrity caused by shift work [26]. As a member of Membrane-Associated Guanylate Kinase (MAGUK) family, *DLG4* is an innate immune receptor that plays pivotal roles in synaptic plasticity [27]. *Rab11* controls the transportation and signal transduction of *DLG4* [28]. It has been widely accepted that synaptic plasticity is closely related to sleep status [29]. A previous study has shown that methylation level of *DLG4* and *Rab11* were altered in mice with sleep disorders, and the changed methylation levels of *DLG4* and *Rab11* were proved to be related to the changes in stress response and circadian rhythm [14]. In our study, methylation levels of *DLG4* and *Rab11* were found to be significantly increased in type 2 diabetes patients combined with somniphathy compared with patients without somniphathy. In addition, transcription level of *DLG4* was positively related to methylation level, and transcription level of *Rab11* was negatively related to methylation level. Those results suggested that changes in transcription profile of *DLG4* and *Rab11* caused by methylation were related to the development of type 2 diabetes in patients with somniphathy, and different methylation patterns may regulate transcription to different directions.

DNA hydroxymethylation also play pivotal roles in regulating genome-wide gene transcription pattern [30]. *Arc*, *Creb3* and *Erg1* are synaptic plasticity genes, and hydroxymethylation levels of *Arc*, *Creb3* and *Erg1* were found to be significantly altered in mice after sleep deprivation [16]. Consistent with previous studies, in our study, hydroxymethylation levels of *Arc*, *Creb3* and *Erg1* in patients with somniphathy combined with type 2 diabetes were found to be significantly higher than those in patients without somniphathy. In addition, transcription

levels of *Arc* and *Erg1* were found to be positively correlated with their hydroxymethylation levels, while transcription level of *Creb3* was found to be negatively correlated with its hydroxymethylation level. Those results suggested that changes in transcription profiles of *Arc*, *Creb3* and *Erg1* caused by DNA hydroxymethylation were related to the development of type 2 diabetes in patients with somniphathy, and the different directions of transcription regulation may be explained by different patterns of DNA hydroxymethylation.

In conclusion, increased methylation levels of *DLG4* and *Rab11*, and hydroxymethylation levels of *Arc*, *Creb3* and *Erg1*, which significantly affected gene expression, were related to the development of type 2 diabetes in patients with somniphathy.

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