Characterization of the 5' Regulatory Region of the Human *RGS4* Gene In Vitro

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Abstract

Background: In order to detect function of three SNPs (rs12041948, rs6678136 and rs7515900) in 5' regulatory region of the human *RGS4* gene, fragments of 5' regulatory region of *RGS4* (-1112–+365, TSS+1) was cloned into pGL3-Basic vector, and dual-luciferase reporter assay was conducted. We compared and analyzed the relative fluorescence intensities of eight haplotype recombined vectors.

Results: In HEK-293 and SK-N-SH cells, the relative fluorescence intensities of haplotype 2 (ATA) was significantly increased when it was compared with haplotype 3 (ACA), 5 (ACC), 7 (GCA), and 8 (GCC). Therefore, haplotypes with C of rs6678136 decreased expression than theses with T. However, no significant difference was assessed in comparation among eight haplotypes, in the U87 cells.

Conclusions: The mutant of T>C of rs6678136 might alter the binding of transcription factors to 5' regulatory region of *RGS4* gene, then change the expression. It was predicated that the rs6678136 might alter the binding region of GSX1, ALX3, BARHL1, and BARHL2. The binding is still worthy of further investigation.

Highlights

1. In HEK-293 and SK-N-SH cells, the relative fluorescence intensities of haplotype 2 (ATA) was significantly increased, compared with haplotype 3 (ACA), 5 (ACC), 7 (GCA), and 8 (GCC).
2. Allele C of rs6678136 decreased the relative fluorescence intensities than T.
3. It was predicated that the rs6678136 might alter the binding region of GSX1, ALX3, BARHL1, and BARHL2. The binding is still worthy of further investigation.

Background

Regulators of G-protein signaling (RGS) negatively modulate G-protein signaling by acting as GTPase-activating proteins. *RGS4* is one important RGS, and associated with many neurologic diseases, such as schizophrenia [1] and neuroglioma [2], addiction, seizure [3] and pain. RGS4 is reported to play an important role for neurotransmission, neuronal differentiation [4] and axonogenesis [5]. *RGS4* gene is located in 1q21-22, which may support *RGS4* gene as an inherent susceptibility factor for the psychiatric illness [6].

There are five transcriptional initiation sites in the *RGS4* gene, producing various protein subtypes [7]. The 5' regulatory region affected the translation, localization and stability of the mRNA [8]. The 5' regulatory region, a primary determinant of translation efficiency, regulated transcription and influenced the expression of protein [9]. The longest variation (RGS4-1) was poorly studied. Association between SNPs of 5' regulatory region in *RGS4* gene and the risk of schizophrenia was widely studied, however, the association was controversial [10, 11]. Our previous study suggested that genotype TT of rs12041948 and GG of rs6678136 and CC of rs7515900 could be risk factors for schizophrenia, in the northern

Chinese Han population. These SNPs might change binding between transcription factors and 5' regulatory region of gene [12]. Transcription factors are recognized as the master regulators of gene expression [13, 14]. Functional studies of the three SNPs are required to confirm these findings.

Methods

Samples

We selected DNA samples containing eight haplotypes (composed with rs12041948, rs6678136 and rs7515900) to construct the pGL3 recombinants. All samples were collected in accordance with the principle of informed consent. All patients and participants provided written informed consent prior to inclusion in this study. Specimens were obtained and analyzed with approval from the Ethics Committee of China Medical University.

Construction of haplotype recombinants in the 5' regulatory region of RGS4 gene

Fragments of 5' regulatory region of *RGS4* gene (-1112–+365, TSS + 1), containing haplotypes 1–8, were amplified with primers (Table 1). The cleavage sites of *NheI* and *HindⅢ* were introduced in the 5' end of the sense and antisense primers, respectively. The target fragments were cloned into the pBM20S vector (Biomed, Beijing, China), and then re-cloned into the pGL3-Basic vector (Promega, Madison, WI), and then verified by DNA sequencing.

Table 1
Primer sequences of the target fragments containing the cleavage sites

Primers	Sequences
5RF	5' CTAGCTAGCGACAAATCAGGCTTCTC3'
5RR	5' CCCAAGCTTCTTTCCTCTTTTGG 3'
Note: Sequences under line was introduced cleavage sites.	

Cell Culture And Dual-luciferase Reporter Assay

The human embryonic kidney (HEK-293) and human glioblastoma U87 were cultured in KeyGEN BioTECH® DMEM in the presence of 10% FBS, and neuroblastoma (SK-N-SH) cell lines were cultured HyClone® DMEM supplemented with 15% FBS (PAN-Biotech). All cells were cultured in a humidified 37 °C environment at 5% CO2. Cells were plated into 24-well plates at 2×10^5 cells per well, and cultured to a density of 90%. The pGL3 recombinant vectors (500 ng) and pRL-TK (50 ng) were transiently co-transfected using Lipofectamine®3000 reagent (Invitrogen, CA). After culturing 30 hours, cell lysates were collected for the reporter assay using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI). The firefly luciferase activity (LUC) and the renilla luciferase activity (TK) were detected,

according to the manufacturer's protocol. Each recombinant was tested in triplicate per experiment with total of three experiments [15].

Statistical analysis

The relative fluorescence intensity was showed as value of LUC normalized by TK. The relative fluorescence intensity differences between each two recombinants were calculated with one-way analysis of variance and Bonferroni's multiple comparison test

using Graphpad prism 7 [16]. A p-value less than 0.05 represented a statistically significant difference in relative chemiluminescence intensities.

Results

Comparison and analysis of relative fluorescence intensities of eight haplotype recombined vectors

Fragments of 5' regulatory region of *RGS4* gene, containing haplotypes 1–8, were constructed into the pGL3-Basic vector, and successfully verified by sequencing. The relative fluorescence intensities of eight haplotype recombined vectors were detected in HEK-293, SK-N-SH, and U87 cell lines (Fig. 1).

In HEK-293 and SK-N-SH cells, the relative fluorescence intensities of haplotype 2 (ATA) was significantly increased when it was compared with haplotype 3 (ACA), 5 (ACC), 7 (GCA), and 8 (GCC); and haplotype 5 (ACC) was significantly lower than haplotype 4 (GTA). In SK-N-SH cells, other significant relative fluorescence intensity differences were found in haplotype 1 (GTC) compared with those in haplotype 3 (ACA), 5 (ACC), and 8 (GCC); in haplotype 3 (ACA) compared with those in haplotype 4 (GTA), and 6 (ATC); in haplotype 5 (ACC) compared with those in haplotype 6 (ATC); and in haplotype 6 (ATC) compared with those in haplotype 7 (GCA), and 8 (GCC). In the U87 cells, no significant difference was assessed in comparation among eight haplotypes.

After analysis, it was detected that the relative fluorescence intensities of haplotypes with C of rs6678136 were lower than these with T, in HEK-293 and SK-N-SH cells.

Results of bioinformatics platform prediction

Transcription factor predictions for the functional fragments were carried out using JASPAR (http://jaspar.genereg. net). The mutant of rs6678136 might influence the binding of transcription factors, such as GSX1, ALX3, BARHL1, and BARHL2.

Discussion

RGS4 protein may negatively regulate G-protein coupled receptors to inhibit the interaction of glutamate, dopamine and other neurotransmitters, resulting in the disorder of above neurotransmitter system [17].

The dysfunction of these neurotransmitter systems is an important pathophysiological feature of psychiatric disorders [5]. *Mirnics et.al* found that only *RGS4* gene expression was consistently altered among 70 genes mapped to the major schizophrenia susceptibility locus 1q21–22, on the microarrays [18]. SNPs in 5’ regulatory region might change transcription and translation of gene [19]. Function of SNPs in 5’ regulatory region of RGS4-1 was poorly studied. On base of our previous study [20], we conducted functional analysis of three SNPs (12041948, rs6678136 and rs7515900).

The relative fluorescence intensities of eight haplotypes, composed with 12041948, rs6678136 and rs7515900, were detected in three cell lines (HEK-293, SK-N-SH and U87). In HEK-293 and SK-N-SH cells, the relative fluorescence intensities of haplotypes with C of rs6678136 were lower than theses with T. Therefore, the transcriptional activity of the 5’ regulatory region fragment carrying C of rs6678136 was the lower than that carrying T. The polymorphism of rs6678136 might influence the binding of some transcription factors to the cis-regulatory elements within 5' regulatory region of *RGS4* gene, and then alter the transcription [21]. In the U87 cells, no significant difference was assessed in comparation among eight haplotypes. The inconsistent finding in the three cell lines was due to different cell microenvironment [22].

It was demonstrated that NF-YA and C/EBP binding to the RGS4-3 promoter [23],and altered the expression of *RGS4* gene. However, there was few studies about character of the promoter of RGS4-1. In our study, it was predicted that this T > C mutation of rs6678136 might reduce ability of transcription factors binding, such as GSX1, ALX3, BARHL1, and BARHL2, and the induction of *RGS4* transcription. It was studied GSX transcription factors controlled neuronal versus glial specification [24], promoted progenitor maturation and the acquisition of neuronal phenotypes [25]. BARHL1 was detected overexpressing in medulloblastoma and played an important role in neurogenesis. BARHL1 was downregulated in Alzheimer's Disease and other psychiatric disorders [26]. The changing binding of these transcription factors by mutation of rs6678136 might induced psychiatric disorders. Further studies were need to warrant the binding of transcription factors and 5' regulatory region of *RGS4* gene.

Conclusions

In HEK-293, SK-N-SH and U87 cells, the mutant of T > C of rs6678136 might alter the binding of transcription factors to 5' regulatory region of *RGS4* gene, then change the expression. It was predicated that the binding region of GSX1, ALX3, BARHL1, and BARHL2 in *RGS4* gene might be altered by the SNP of rs6678136. The binding is still worthy of further investigation.

Abbreviations

RGS

Regulators of G-protein signaling.

Declarations

Ethics approval and consent to participate

All samples were collected in accordance with the principle of informed consent. All patients and participants provided written informed consent prior to inclusion in this study. Specimens were obtained and analyzed with approval from the Ethics Committee of China Medical University.

Consent for publication

All authors have read and approved the manuscript for submission.

Availability of data and materials

This was an evidence synthesis study; all data were available.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

BW designed the study and wrote the protocol. FX managed the study, which was checked by YL, MD, JX, JX. FX performed analyses. The manuscript was written by FX, and corrected by JY.

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Figures

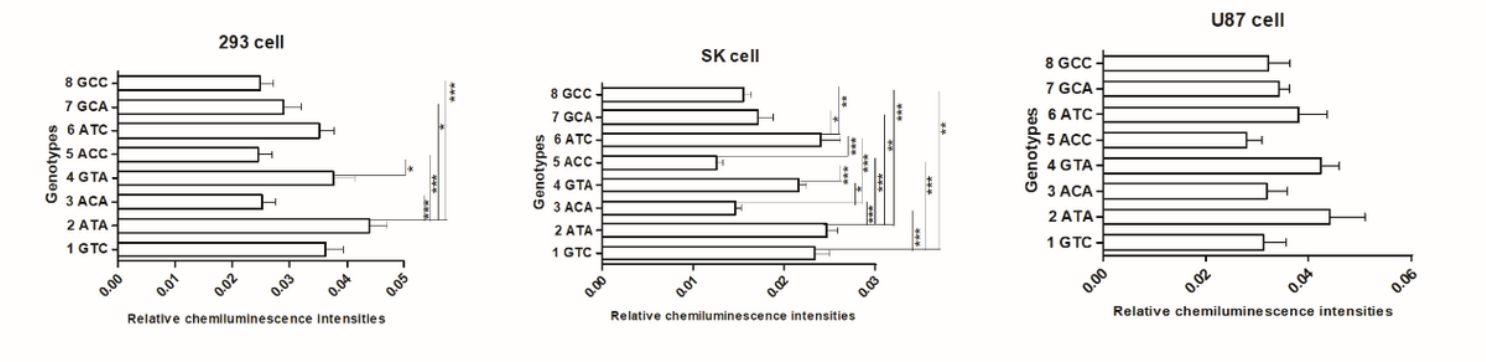

Figure 1

Relative fluorescence intensities for eight haplotype recombinants in HEK-293, SK-N-SH and U87 cells. * $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.001$