Effect of CpG Sites on Transcription Factor in Promoter of 'RASA3' Gene

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ABSTRACT

RAS p21 activator 3 gene is a member of the Ras GAP1 family of GTPase-activating proteins. DNA methylation is the most well-known epigenetic mechanism. Abnormality on RAS proteins which are regulated by *RASA3* may play a critical role in Autism. Autism is a complex of neurodevelopmental disorders. Defining the regulators of *RASA3* is important to understand its role in Autism. This study included 19 Saudi autistic children and their healthy siblings. Blood was used to analyze the differential expression of *RASA3* and its transcriptional factor Specificity protein 1. qRT-PCR and MethyLight assay was used to measure CpG sites methylation on TFBs in the promoter of *RASA3* to investigate the association between methylation level and TF in the regulation of *RASA3* expression. Results showed differential expression patterns for both *RASA3* and *Sp1* genes with a significant positive correlation between them. There was no difference in *RASA3* methylation level in such binding sites of the target region in the promoter of *RASA3* in autistic and healthy samples. The data suggested that the Sp1 could play a critical role in the regulation of *RASA3* expression while DNA methylation does not. However, it remains an open question whether the dysregulation of *RASA3* by methylation in other regions may be implicated in the development of ASD.

Key Words: *RAS o21, Protein activator, Proliferation, Transcription, Autism*

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INTRODUCTION

RAS p21 protein activator 3 (*RASA3*) is a member of the Ras GAP1 family of GTPase-activating proteins known as dual GAP for Ras and Rap-GTPases. RASA3 is a negative regulator of Ras proteins which play an important role in fundamental cellular mechanisms such as proliferation and transcription [1]. The expression of *RASA3* was found at the highest levels in the brain tissues (neurons and oligodendrocytes), where its expression increased with development [2]. The expression of *RASA3* can be regulated through Transcription Factors (TFs) and DNA methylation. DNA methylation is a highly well-known epigenetics regulation [3]. Hypermethylation on CpG sites in the promoter region is thought to prevent gene expression by disrupting the binding between TFs and Transcription Factor Binding sites (TFBs) [4-6]. However, much uncertainty still exists about the relation between

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aberrant methylation and TFs. In recent years, researchers found that methylation aberrations can cause alterations in gene expression and develop many common diseases such as tumors, schizophrenia (SZ), and Autism Spectrum Disorder (ASD) [7-9].

Both the methylation status and differential expression of TFs of *RASA3* were shown to be implicated in several diseases. Subtelomeric deletion of chromosome 13 including the *RASA3* gene has been associated with schizophrenia [10]. Besides, several studies showed that aberrant methylation in *RASA3* may be associated with Hepatocellular Carcinoma (HCC) development and schizophrenia disorder [11, 12]. Furthermore, RASA3 has been shown to affect many biological processes such as proliferation and transcription by negatively regulating Ras proteins which are binary switches during the signal transduction pathway. Previous work on Ras proteins found that Ras implicated in some neurological disorders, such as ASD, and any abnormalities on Ras proteins may play a critical role in ASD and other neurological disorders [13]. The correlation between schizophrenia and ASD is highly considerable since they both are neurodevelopmental disorders that overlap in some clinical features including psychiatric, communication, and cognitive impairments [7, 14-16]. Therefore, the study of DNA methylation sites and TFs that contribute to the relation between *RASA3* expression and ASD is important. ASD is a complex group of neurodevelopmental diseases characterized by problems with social communication, language and speech impairments, and restricted repetitive behaviors [17]. ASD is increasingly recognized as a serious, worldwide public health concern, and the etiology is still unknown. Although environmental and genetic factors are found to be influencing ASD but are still not well understood [18]. DNA methylation may provide interference between genetics and environmental factors, and in recent years, there has been an increasing amount of literature investigating the role of aberrant DNA methylation in ASD.

Collectively, the previously-mentioned studies which have reported that differentially expressed *RASA3* and its methylation level is correlated with multiple disorders, have provided support for the potential role of CpG sites methylation and TFs in gene expression regulation, which attracted our attention to understand the effects of CpG sites methylation on transcription factor binding sites in the regulation of *RASA3* expression which could be associated with Saudi autistic children.

MATERIALS AND METHODS

Study population

A total of 37 samples (19 ASD children and 18 of their healthy siblings) aged from 3 to 12 years old were subjected to this study. Children who are suffering from malnutrition, active infection, or known genetic disease (such as Down syndrome) were excluded. All the patients were Saudi. The study was approved by the ethical committee of the Center of Excellence in Genomic Medicine Research (CEGMR) and informed consent was obtained from parents or guardians of all children. Blood samples were collected by the Center of Excellence in Genomic Medicine Research (CEGMR) as well as from the pediatric clinic in Jeddah. Venous blood was collected into 3-ml EDTA anticoagulant tubes and then stored at −80 °C.

Quantitative real-time RT-PCR

Total RNA was isolated from peripheral blood using RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. First-strand complementary DNA was synthesized from total RNA with ImProm-II Reverse Transcription system kit (Promega, Madison, WI USA), according to the manufacturer's instructions. Subsequently, aliquots of cDNA were subjected to PCR by using the QuantiFast SYBR® Green PCR Kit (Qiagen, Hilden, Germany) on Applied Biosystems™ StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, California). Primers for mRNA detection of RASA3, Sp1, HBA1 and β-actin (internal control) were as follows: for RASA3, 5'-AAACCTTCCCTCTTACCCGG-3' (forward) and 5'-TGA CGA AAG CTC CGA GGAAT-3' (reverse); for Sp1, 5'-AGTTGGTGGCAATAA TGGGG-3' (forward) and 5'-CTGGGAGTTGTTGCTGTTCT-3' (reverse); for HBA1, 5'-CGGTCAACTTCAAGCTCCTA-3' (forward) and 5'-AACGGTATTTGGAGGTCAGC-3'; for β-actin, 5'-AAAATCTGGCACCACACCTT-3' (forward) and 5'-GCCTGGATAGCAACGTACAT-3' (reverse). The amplification conditions were as follows: 95˚C for 5 min, followed by 40 cycles of 95˚C for 10 sec. and 58˚C for 30 sec. The expression levels of target genes were calculated using the 2^-ΔΔCt method normalized to β-actin.

DNA extraction and bisulfite conversion

Genomic DNA was isolated from peripheral blood using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Genomic DNA was converted through sodium bisulfite treatment using EpiTect Bisulfite Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Methylight assay

The methylation level of RASA3 was analyzed using the EpiTect MethyLight PCR Kit with TaqMan probes (Qiagen, Hilden, Germany). The MethyLight assay for RASA3 methylation was performed using the probe FAM-TCGTTATTCGTTAGCGTCGT-BHQ1 and amplification oligonucleotide primers RASA3-MLF 5'-GATTTTTGCGTTTTTCGGGTT-3' and RASA3-MLR 5'-CTACAAAACCGCCAATTAAC-3'. For normalization, a probe (HEX-CCTTCATTCTAACCCAATACCTATCCCACCTCTAAA-BHQ1) targeting methylation independent and bisulfite-conversion-dependent COL2A1 sequence was used with the amplification primers COL2A1-CTRL-F (5'-TCTAACAATTATAAACTCCAACCACCAA-3') and COL2A1-CTRL-R (5'-GGGAAGATGGGATAGAAGGGAATAT-3').

Promoter sequencing

Gene-specific primers (RASA3-F/R) were designed to amplify an 800-base pair (bp) promoter region upstream of the RASA3 transcriptional start site. The genomic DNA was amplified by the Polymerase Chain Reaction (PCR) technique using a Go Taq Green Master Mix (Promega, Madison, USA). The primers were: 5'-ATGCCGGAGGTCTGGGGT-3' (forward), 5'-TGGCTGCCGGCGGACACT-3' (reverse). The amplification conditions were as follows: 95˚C for 10 min, followed by 30 cycles of 95˚C for 15 sec., 58˚C for 30 sec. and 70 for 30 sec., followed by final 70˚C for 10 min. The PCR product was extracted from an agarose gel. Sequencing was performed in both directions by Sanger's dideoxynucleotide chain termination method using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystem, Graiciuno, Lithuania), according to the manufacturer's instructions.

Statistical analysis

The IBM SPSS® Statistics (IBM Company, New York, NY, USA) software package (IBM SPSS Statistics, version 20) was used to perform the statistical analysis. A p-value <0.05 was considered statistically significant. Pearson correlation was used to evaluate the linear relationship between the two variables. It ranges from +1 to -1 which +1 means a strong positive relation and -1 indicates a negative relation between the two variables.

RESULTS AND DISCUSSION

Methylation and transcription factor binding sites prediction

The analysis of the 800 bp of the *RASA3* promoter region by MethPrimer software shown one CpG island in the length of 575 bp located in (170/744). We behaviors and difficulties with language and speech. While the prevalence increased during the last years, the etiology is still unknown. There is a considerable correlation between ASD and schizophrenia. *RASA3* was found to play a role in several diseases such as cancer and schizophrenia. Therefore, *RASA3* could play a critical role in ASD. We have previously shown that Sp1 (Specificity protein 1) is one of the top transcription factors that affect the expression of *RASA3*. Sp1 is a member of the SP family of zinc finger (Cys2/Hys2) where TFs attaches to the GC-box promoter [19]. Sp1 regulates the expression of housekeeping genes as well as many different genes in the brain. Therefore, Sp1 plays a vital role in several cellular functions, such as cell differentiation, chromatin remodeling, and immune response and any alteration in its function will produce dysregulation in its target gene expression [20, 21]. A recent study has found that upregulation of *Sp1* in the autistic brain changes the expression of some autism-associated genes [22]. Other recent studies have considered that DNA methylation could inhibit the binding of TFs such as Sp1 [23, 24]. DNA methylation plays an important role in gene expression by turning the gene on and off [25]. Aberrant methylation caused alteration in the gene expression and therefore several diseases such as ASD and cancers [26]. Furthermore, methylation may affect the association between TFs and the binding sites, but the impact differs between TFs [6]. Therefore, our hypothesis suggests that aberrant methylation can be associated with the differences in the expression level of *RASA3* and its potential transcription factors in ASD brain function.

In the presented study, we aimed to quantify the methylation and expression level of *RASA3* and analyze the correlation between *RASA3* and its potential transcription factor (*Sp1*) in 19 Saudi autistic children comparing to their control siblings. We hypothesized that aberrant CpG methylation could affect the transcription factor binding sites and thereby the correlation between *RASA3* and its potential transcription factors in ASD.

Our findings indicated that differential expression patterns were observed for both *RASA3* and *Sp1* normalized with *β-actin* endogenous control gene. This differential of expression shows an increased probability of the role of the *RASA3* in ASD. Although the nature of this role is still unknown, it may affect ASD through its relationship with *Sp1*. We observed a significant positive correlation between the expression of *RASA3* and *Sp1* (Pearson's r=0.709; p=0.001). This finding is consistent with those of Thanseem *et al*. (2012) who found that overexpression of the

transcription factor *Sp1* alters the expression of potential autism candidate genes [22]. Another important finding was that a difference in the DNA methylation level in the autism and normal samples were not detected by MethyLight assays in the target region. Furthermore, sequencing analysis results showed that there was no mutation in the predicted binding sites. While such region was selected based on Sp1 TFBSs predicted data, another potential methylated region could be detected by using a genome-wide DNA methylation analysis to investigate the level of DNA methylation in the whole *RASA3* promoter.

CONCLUSION

The presented study indicated that *RASA3* and *Sp1* expression showed differential levels in autistic patients. Furthermore, there was no difference in *RASA3* methylation level in such candidate binding site of *RASA3* promoter in autistic and healthy samples. The presented data suggest that the Sp1 transcription factor can play a critical role in the regulation of *RASA3* expression while DNA methylation cannot, at least in the region included in this study. However, it remains an open question whether the dysregulation of *RASA3* by methylation in other regions may be implicated in the development of ASD. Therefore, further study is required to elucidate this correlation.

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Conflict of interest: None

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Ethics statement: None

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