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The Correlation between Gene Expression and DNA Methylation Levels of RAS p21 Protein Activator 3 (*RASA3*) Gene in Saudi Autistic Children

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SA, AA and RA designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors SA, AA, MA, KA, RF, FB, MS and AD managed the analyses of the study. Authors NA, MG, HA and MJ managed the literature searches. Authors AE, HA, SA and AA revised and edited the final draft. All authors read and approved the final manuscript.

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ABSTRACT

The potential role of DNA methylation pattern in autism has been provided by revealing the differences in methylation level of multiple genes which are significantly associated with their expression and implicated in ASD pathogenesis. *RASA3* is a member of GTPase-activating

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proteins, *RASA3* is highly expressed in brain tissues and can be deregulated by different epigenetic mechanisms. Many studies reported that differentially expressed *RASA3* is correlated with its aberrant methylation. Accordingly, this has been suggested that deferentially expression of *RASA3* may be correlated with its methylation levels which could play a role in ASD which brought our attention to identify differentially-expressed genes that could be associated with their methylation level of ASD in Saudi population, by performing comparative gene expression of *RASA3* then investigate its relation to methylation level.

This study was conducted on 18 Saudi autistic children as well as their healthy-control siblings. Relative expression of a candidate gene (*RASA3*) was measured using RT-qPCR. Furthermore, MethyLight assay was performed to estimate methylation level and evaluate its impact on *RASA3* expression. Interestingly, *RASA3* expression has found to be dysregulated in ASD cases. In contrast, MethyLight assay result showed no differences in the methylation patterns among ASD cases in the candidate region. However, it remains an open question whether these dysregulations of *RASA3* expression could be a biomarker for early screening/detection of some cases which may also suggest a role for RasGAPs in autistic brain function.

Keywords: DNA methylation; Autism; ASD; RASA3.

1. INTRODUCTION

1.1 Autism

Autism spectrum disorder (ASD) is a complex of neurodevelopmental disorders with early age of onset, typically before three. ASD diagnosis is based on comprehensive behavioral assessments and it is characterized by pervasive behavioral regressions, language deficits, stereotyped behaviors as well as social interaction impairments. Several ASD patients have suffered from other problems than the core symptoms of ASD including attention deficits and hyperactivity disorder (ADHD), epilepsy, gastrointestinal conditions and sleep disturbances [1-3]. The prevalence of ASD has rapidly risen over the past several decades. following this progressive increase of ASD cases worldwide and lack of efficient diagnosis and treatment, in addition to the difficulties on the society and families of autistic children, the research and investigation of ASD biomarkers became a fundamental priority. Regarding the statistical report in the 2017 disability survey which reported by the General Authority for Statistics and King Salman Center for Disability Research, the prevalence of autism has raised over 50,714 autistics [4]. It is reasonable to mention that there are some arguments on whether this increase in occurrence is due to the broadening and enlargement of diagnostic criteria, awareness, screening, or increasing exposure to environmental risk factors.

1.2 Epigenetic of ASD

The causes of ASD are heterogeneous and still indistinct, this includes environmental factors, chromosomal abnormalities, and mutations. Numerous studies have been designed to demonstrate the etiology of ASD through genetic mutations with less emphasis on the potential role of the epigenetic and environmental causes because of the high heritability estimates in autism. However, the correlation linking ASD and environmental factors have been widely investigated. According to multiple studies, genetic and environmental factors are both implicated in autism pathogenesis [5-8]. DNA methylation is one of the most common epigenetic mechanisms which has been mentioned in several studies to investigate the correlation between ASD and epigenetic modifications, since genetic and epigenetic are both involved in ASD. However, several studies have investigated the epigenetic architecture of ASD by studying DNA methylation since it plays a major role in the interface between genetic and environmental factors. Notably, a strong association between autism and methylation patterns has been considered and reported in numerous studies. MECP2 is considered to be a significant epigenetic regulator in human brain development and highly enriched in the nervous system, DNA methylation modifications in the promoter region of MeCP2 were revealed in autistic brains linking autism [9,10]. MECP2 is also a member of the methyl-binding domains which binds to methylated cytosine and acting as an activator or a repressor to the gene. Numerous studies have investigated the binding of MECP2 and its role in the expression regulation of multiple genes. A study has reported that MECP2 is significantly increased binding specifically to the promoter regions of GAD1 and RELN in the cerebellar cortices of ASD patients, this study found that increasing

binding of MeCP2 protein to the RELN promoter as well as GAD1 promoter was negatively correlated with of RELN and GAD1 mRNA expression in ASD [11].

FOXP1 gene expression is discovered to be associated with the pathogenesis of ASD which could be regulated by its methylation patterns. Another two studies have compared methylation patterns with the expression level of ENO2 gene and RELN gene, showed hypermethylation in the promoter region and suggested that downregulation expression of these genes could be a biomarker for autistic children [12-15]. Since epigenetics is a rapidly growing field, methylation studies in neurological diseases such as ASD promise to be a thrilling field of research that will open up new ways for facilitating early screening, detection, and treating neurological diseases.

1.3 The Potential Role of RASA3 Methylation Patterns Influencing Gene Expression

RASA3 is a member of GTPase-activating proteins (Ras GAPs), these proteins play several important roles through regulating the G protein activity since G proteins are the most essential mediators of extracellular signals and intracellular effectors involved in a variety of key intracellular processes. Ras protein is involved in essential cellular processes such as proliferation and transcription process. *RASA3* has a double specificity GTPase activating protein which can stimulate the GTPase proteins activity of RAS and RAP depending on their cellular situation [16]. Methylations patterns in RASA3 plays a significant role in the regulation of gene expression, numerous studies have been established to investigate the correlation between RASA3 methylation and its expression levels in multiple diseases. RASA3 was found to be differentially methylated in hepatocellular carcinoma (HCC), diabetic embryopathy (DE) and serve as a useful biomarker that became a promising epigenetic biomarker for early detection and progression [17,18].

Although, up till now there isn't any study has directly investigated the relation between RASA3 methylation level in ASD comparing to normal cases, the correlation between SZ and ASD is highly considerable since they both are neurodevelopmental disorders overlaps in some clinical features including psychiatric, communication and cognitive impairments. A methylomic profiling study of human brain tissue from patients with schizophrenia (SZ) has found that RASA3 is one of the most significant genes which found to be differentially methylated in patients compared with controls [19,20]. Collectively, the previous-mentioned studies have reported that differentially expressed RASA3 is correlated with its aberrant methylation levels. This provided corroboration for the probable role of DNA methylation pattern in gene expression levels, which definitely concerned our attention to identifying an innovative differentially expressed gene that could be associated with the methylation level of ASD in Saudi Arabia, using relative gene expression of RASA3 then study its relation to their methylation levels.

This study aims to investigate the correlation between differential expression of RASA3 gene association with its DNA methylation levels of autistic children in Saudi population comparing with their healthy control sibling. RASA3 expression level in ASD patients and their siblings were measured using RT-qPCR and then compared to its methylation level which measured using MethyLight qPCR assay.

2. MATERIALS AND METHODS

2.1 Study Population

A total of 36 children aged between 3 to 12 years old were subjected to this study, 18 Saudi autistic children (doctor diagnosed), 17 boys and one girl, as well as their 18 healthy control siblings. Subjects were medically diagnosed with ASD in a specialized clinic in Jeddah. All subjects were recruited in this study have not suffered from malnutrition and no other known genetic diseases or any active infections. Each participant's family has a written agreement for conducting their children into this study and has filled the research questionnaire. 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 EDTA anticoagulant tubes by the CEGMR as well as from the pediatric clinic in Jeddah and then stored at −80 °C for gene expression analysis.

2.2 RNA Extraction and cDNA synthesis

Gene expression level analysis of *RASA3* was measured using RT-qPCR. Total RNA was extracted from a whole blood sample using (RNeasy Mini Kit, Qiagen). In preparation for RNA quantification by real-time qPCR, the first-

strand cDNA was synthesized from total RNA using (GoScript™ Reverse Transcription System kit, Promega).

2.3 RT-qPCR

For PCR amplification of cDNA samples, the primer sequences of *RASA3*, as well as endogenous β-actin (*ACTB*) gene were designed using NCBI Genome Browser to get the sequence of the interested genes. Primers for mRNA detection of *RASA3* and *ACTB* were as follows: for *RASA3*-F *(*5'-AAA CCT TCC CTC TTA CCC GG-3') and for *RASA3*-R (5'-TGA CGA AAG CTC CGA GGA AT-3'); for *ACTB*-F (5'-AAA ATC TGG CAC CAC ACC TT -3') and for *ACTB*-R (5'- GCC TGG ATA GCA ACG TAC AT -3').

StepOne Plus Real Time PCR machine and StepOne software v2.3 (Applied biosystem) was used for relative gene expression quantification to determine expression levels of *RASA3*. The reaction was carried out using (Fast SYBR™ Green Master Mix kit, Thermo Fisher), the expression level of *RASA3* was expressed relative to the housekeeping gene *ACTB* which was used as an endogenous control gene. Two PCR replicates were carried out for each sample and used to calculate the mean cycle threshold (Ct) value. The expression levels of *RASA3* was normalized to *ACTB* and calculated using the 2-ΔΔCt method [21], Pfaffl equation which used to determine the relative amount of mRNA of the target gene in different samples.

2.4 DNA Extraction and Bisulfite Conversion

Methylation level analysis of *RASA3* was measured using MethyLight qPCR, this procedure conducted by carrying out DNA extraction and bisulfite conversion, MethyLight qPCR**.** Blood samples in EDTA tubes (Lavender-top tube) were inverted several times to avoid any precipitations or clotting. Genomic DNA was extracted from whole blood samples using DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany) according to manufacturer's instructions. The ability to detect and quantify DNA methylation via MethyLight technique requires the starting template to be converted such that the methylated cytosines can be distinguished from unmethylated cytosines. Genomic DNA was converted through sodium bisulfite treatment using EpiTect Bisulfite Kit (Qiagen, Hilden, Germany).

2.5 Methy Light qPCR

MethyLight primers and probes were designed for both the *RASA3* as well as Collagen (*COLA*) which was used as an endogenous control for MethyLight qPCR assay. Eukaryotic Promoter Database EPD (https://epd.epfl.ch//index.php) was used to access the database of promoter sequences. Sequence Retrieval Tool was used to specify the targeted region from the promoter, the exact length of the promoter was selected in the region from 600 bp upstream to 200 bp downstream of the TSS.

Primers and probes for methylation analysis of *RASA3* and *COLA* were as follows: for *RASA3* probe (FAM 5'-TCG TTA TTC GTT AGC GTC GT-3' BHQ1) and amplification oligonucleotide primers, *RASA3*-ML-F (5'-GAT TTT TGC GTT TTT CGG GTT-3') and for *RASA3*-ML-R (5'-CTA CAA AAC CGC CAA TTA AC-3'). For the internal control and normalization, *COLA* probe (HEX 5'-CCT TCA TTC TAA CCC AAT ACC TAT CCC ACC TCT AAA-3' BHQ1) targeting the methylation independent and bisulfite-conversion-dependent *COLA* sequence was used with the amplification oligonucleotide primers *COLA*-ML-F (5'-TCT AAC AAT TAT AAA CTC CAA CCA CCA A-3') and *COLA*-ML-R (5'-GGG AAG ATG GGA TAG AAG GGA ATA T-3').

The methylation analysis of *RASA3* was then analysed using (EpiTect MethyLight PCR Kit, Qiagen according to the manufacturer's instructions. MethyLight was conducted for quantitative methylation analysis using probe-based real-time PCR for methylation analysis to determine the relative prevalence of a particular pattern of methylated CpG dinucleotides among ASD patients. The reactions were carried out using StepOne Plus Real Time PCR machine (Applied biosystem).

3. RESULTS

In this study, the dysregulation of *RASA3* between autistics and controls shows that in 61.11% of the cases, gene expression was downregulated, while in 38.88%, *RASA3* was upregulated. Overall, a highly significant decrease was reported in the expression of *RASA3* in 8 ASD cases that gave (-102.39, -57.83, -14.11, -6.99, -4.12, -3.32, -2.59, -2.50)-fold change, while another 3 ASD cases show moderately decrease (-1.49, -1.41, -1.17)-fold change which represents the downregulation in the target gene. In contrast, a significant increase

in the expression of *RASA3* in 5 ASD cases, which yields (2.91, 7.72, 13.57, 60.83, 3706.82)-fold change, while two shows moderately increase (1.38, 1.46)-fold change which represents the upregulation in the target gene. To this end, the results of RT-qPCR reflect variations in the expression levels in the cases, which could be attributed to the variation in the cellular composition of blood samples, gender, or age. However, these changes could be explained by the comparison to the methylation pattern of the target gene *RASA3*. From this work findings, it can be suggested that autism aetiology can be attributed, to some extent, to *RASA3* downregulation in some autistic individuals. Further investigation is certainly needed to elucidate the implications of upregulated *RASA3* as well. Generally, it might be early to state a slid hypothesis regarding the effect of *RASA3* dysregulation in the aetiology of ASD.

MethyLight qPCR was used in this study to determine the relative prevalence of a particular pattern of methylated CpG dinucleotides in *RASA3* promoter region (114132423 - 114133223) bp among autistics and controls. Two sets of primers and probes were designed specifically for bisulfite-converted DNA in this study for both (*RASA3, COLA*). Collagen was used as the endogenous internal reference to normalize for the amount of input DNA. The specificity of MethyLight PCR reaction was assessed by including control reactions to ensure that MethyLight PCR probes and primers will specifically bind, also to exclude any false-negative results. The three DNA sequences were: 100% methylated converted DNA which was used as the positive control, as well as the 100% unmethylated converted DNA and 100% unmethylated unconverted DNA which was used as negative controls. No amplification signal in the negative controls was detected, which confirmed that any negative reaction is not a false negative result. In contrast, the positive control 100% methylated converted DNA showed a normal amplification curve which indicates that the primers were designed specifically to bind and detect only the converted methylated CpG sites in the sequence.

4. DISCUSSION

As this study moves beyond the scope of genetics into transcriptomics and epigenomics, the sample type used in this study was the fresh-drawn blood, since there are several limitations to the use of fresh brain tissue from living ASD patients. Moreover, using brain tissue for diagnostics is impractical way at the current time. Several studies have supported the usefulness of blood cells in gene expression studies as substitute for brain tissue for diagnosing neuropsychiatric disorders [22,23].

Since the methylation patterns of a specific DNA sequence could be affected by multiple factors, the study population in this research was restricted with multiple features such as: nationality, age, and any other health problems. In this research, 36 children from 3 to 12 years were involved since the ASD symptoms such as: pervasive behavioral regressions, language deficits, stereotyped behaviors as well as social interaction impairments which are usually diagnosed and detected at the first years of life [6,24].

The number of studies that compared differences in gene expression between cases and controls for complex disorders is growing. In this investigation of the gene expression on ASD blood samples, we have established differential levels of *RASA3* expression between ASD patients and their siblings. The analysis of RT-qPCR in this study indicated differential expression patterns that were observed for *RASA3* normalized with *ACTB* endogenous control gene.

In an ongoing research project by Alhazmi, which was the first study that reported the correlation between gene expression and its methylation patterns among Saudi autistic children by utilizing DMC (Differentially Methylated Cytosine) analysis in the CpG context. In Alhazmi research, multiple genes reported significant methylation patterns differences between the two samples (ASD and control). Multiple genes were reported significant differentially methylated CpG islands regions, *RASA3* is one of the genes that found to be highly methylated in the autistic child in comparison with its control. The expression level of the methylated *RASA3* was then evaluated by utilizing qRT-PCR which was found to be downregulated in the autistic sample comparing with the control. One of the outcomes of the aforementioned study is the significant difference in methylation patterns of *RASA3* between two siblings which correlated well with differential gene expression, which was hypothesized to contribute to autism etiology.

Considering that MethyLight assay is a methylation-specific PCR approach and thus it is

limited only to the CpGs that lie among the region that the probe is designed to bind, which was only 4 CpGs in this study. The result shows essentially no methylation in the selected area for both control and patient samples. This result may call into design another primers and probe to perform another MethyLight qPCR that including another CpGs area. On the other hand, performing another technique such as pyrosequencing is a good recommendation for assessing the methylation level in a wide range area. When considered together, the sample size was also relatively small. Therefore, if the sample size was larger, the outcome of the analysis would possibly be more conclusive, or at least there could be another explanation for the relationship of *RASA3* expression dysregulations with ASD etiology. Thus, for future work and further investigations it is recommended to conduct an expanded sample of ASD patients.

Despite the negative results of Methylation analysis, the dysregulation in the expression of *RASA3* could explain and indicate RAS protein function in ASD. Therefore, the methylation level of *RASA3* should be further investigating using different molecular techniques.

5. CONCLUSION

The identification of DNA methylation differences among ASD children is a powerful tool that could help assessing the biological mechanism and therapeutic targets for ASD. Our findings support the hypothesis that dysregulation of *RASA3* expression levels may associate with ASD pathogenesis. Although, these variations in the expression levels of *RASA3* in ASD cases could be related to the genetic or environmental factors or due to the variations in the cellular composition of the blood sample, gender, or age.

As MethyLight assay can only reveal the methylation patterns in specific CpG regions, further analysis must be conducted to comprise different CpG sites in order to evaluate the methylation level in a wide-range and compare them to the healthy-siblings. However, it remains an open question whether these expression dysregulations possibly will be used as a biomarker for early screening/detection of some cases which may also suggest a role of RasGAPs in autistic brain function.

This work findings pave the path for more studies for more extensive studies for the Saudi population utilizing a larger sample size and more tightly-controlled investigations with more technical replication and optimization.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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CONSENT

As per international standard, parental written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

This study was approved by the ethical committee of the Center of Excellence in Genomic Medicine Research (CEGMR) no. 02-CEGMR-Bioeth-2018.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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