Association Between RAS-Like Proto-Oncogene B *(RALB)* Gene Expression and Methylation Levels in Saudi Autistic Children

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ABSTRACT

RAS like proto-oncogene B (*RALB*) gene is located in human chromosome 2 and encodes a GTP-binding protein that belongs to RAS subfamily of the small GTPase superfamily. Recently, RAS signaling pathway has been studied as likely related to ASD. ASD is a complex range of neurodevelopmental disorders. DNA methylation (DNAm) is the most studied epigenetic mark in the mammalian genome. Investigatating the expression level of *RALB* gene in autistic patients and assessing the relationship between the expression and methylation level of the *RALB* gene is important to understand its role in Autism. This study included 19 Saudi autistic children and their healthy siblings. Blood was taken to analyze the differential expression of *RALB* qRT-PCR, and MethyLight assay was used to measure the methylation of CpG sites in the promoter of *RALB* in order to investigate the association between methylation level and the expression level of *RALB.* The results showed differential expression patterns for the *RALB* gene. There was no difference in *RALB* methylation level of the target region, i.e. the promoter of *RALB* between autistic and healthy samples. The data suggested a potential role of *RALB* dysregulation in ASD pathogenesis in the Saudi population. However, questions about the role of RALB in the autism etiology and the probability of *RALB* expression dysregulations to use as a biomarker for early diagnosis of some ASD cases remain unanswered at present.

Key Words: *Gene expression, RALB, Autism, ASD, DNA methylation*

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NTRODUCTION

RALB gene is a member of Ral GTPases superfamily, which participates in distinct cellular events, and plays a key role in neuropathological conditions related to ASD [1]. *RALB* is generally expressed in various tissues but has been found to exhibit its highest expression levels in the brain [2]. RALB protein is involved in a number of signaling cascades and affects an assortment of downstream pathways by interacting with a varied array of effectors such as *Ral BP1*, phospholipase D, sec5, and filamin [3, 4]. Many studies have already proved the role of RALB in the neurosecretion, regulation of vesicular trafficking and inducing neurite branching in cortical and sympathetic neurons via their exocyst binding pathways. These findings are consistent with the fact that *RALB* exhibits several features that are predictable of a gene, which might regulate synaptic vesicle trafficking. It is

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supplemented in the synaptic vesicle fraction and recognized to serve as molecular switches, that can be triggered by many signaling cascades [3]. A large body of literatures has linked *RALB* dysregulation to numerous oncogenic activities such as cellular migration and vesicle trafficking, and the signal transduction pathways of this important cellular process strongly overlapped with autism. Previous findings have revealed that the signal transduction pathways of cancer extensively overlap with autism [5]. The expression of RALB can be regulated by DNA methylation. Recently, there has been a growing interest in detecting epigenetic markers and explaining the impact of epigenetics in ASD. Some epigenetic changes including hypo and hyper DNA methylation have been found in the brains of autistic patients, due to some environmental risk factors, including nutrition, stress, maternal care behavior, or toxins [6, 7]. Recently, many researchers reported that aberrant methylation can cause alteration in the expression levels in many genes implicated in ASD [8-11]. Therefore, the study of the correlation between the DNA methylation level and the expression level of RALB in ASD is important. ASD identified by diagnostic criteria includin impaired social interactions, language and communication deficiencies, limited interest in activities, and extremely stereotypical repetitive patterns of behaviors [12, 13]. In the last two decades, the prevalence rate of autism have increased intensely around the world including the middle east and Saudi Arabia. Generally, the etiology of ASD is assumed to implicate a collective interaction of genetic and epigenetic factors. Though, the association between gene expression and methylation levels has not been studied yet in Saudi autistic children. The main object of this study was to estimate the expression level of *RALB* and its association with the methylation level in the Saudi autistic children population, and the experimental work was designed to utilize the qRT-PCR and MethyLight assay.

MATERIALS AND METHODS

Study population

This study was approved ethically by the Center of Excellence in Genomic Medicine Research (CEGMR). Blood samples were collected from the Center for Autism Research (CFAR) at King Faisal Specialist Hospital & Research Center (KFSH&RC) in Riyadh and the padiatric clinic in Jeddah. Study samples included 19 Saudi autistic children (physician diagnosed) at ages 3 to 12, as well as their 19 healthy children in the family (sibling) for comparison analysis. Individuals on medication, or who suffers from malnutrition, presence of active infection or known genetic disease were excluded from the study. Blood sample (2 ml) was withdrawn from the vessel in the arm of both autistic child and his/her sibling only once by a lab technician in the laboratory of King Abdulaziz University Hospital. Then, the sample was collected in anticoagulant EDTA tubes.

Quantitative real-time 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 the total RNA using ImProm-II Reverse Transcription system kit (Promega, Madison, WIUSA). 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 RALB and β-actin (internal control) were as follows: for RALB, 5'-TATGACCTGTGAAGGCTGCA-3' (forward) and 5'-ATCATCTCCCGCTTCCTCTG-3' (reverse); 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 at 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) and 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 was performed for RALB methylation using the probe FAM-TTTTAGTTTTGGTTTGAATTTTAT-BHQ1 and amplification oligonucleotide primers RALB-MLF 5'-TTGTATGATTAGGTTTGTTTTT-3' and RALB-MLR 5'-CAACTAATAACCCTACGACA-3'. For normalization, a probe HEXCCTTCATTCTAACCCAATACCTATCCCACCTCTAAA-BHQ1) targeting methylation independent and bisulfite conversion- dependent COL2A1 sequence was used with the amplification primers COL2A1-CTRL-R (5'-GGGAAGATGGGATAGAAGGGAATAT-3') and COL2A1-CTRL-F (5'-TCTAACAATTATAAACTCCAACCACCAA-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 PCR technique using a Go Taq Green Master Mix (Promega, Madison, USA). 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 °C for 30 sec., followed by 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.

RESULTS AND DISCUSSION

Inclusively, a frequently significant increase was reported in the expression of *RALB* in four ASD cases, and showed 29.9, 11.3, 8.6, 4,92-fold increase. While other seven ASD cases reveal moderate increase expression by 3.0, 2.8, 2.8, 2.6, 1.74, 1.41, 1.41-fold, which represents the upregulation in our target gene. In contrast, a significant decrease was dwtwcted in the expression of 6 ASD cases and showed 4.9, 2.64, 1.9, 1.31, 1.23, 1.2-fold decrease, that characterizes the downregulation in our target gene.

According to many recent studies, *Bcl-2* is a membrane-bound protein that has a neuroprotective function by inhibiting apoptosis and enhancing the survival of neurons in the central nervous systems. Investigation in the autism field revealed that the *Bcl-2* levels are reduced by 36% and 38% in autistic superior cerebellar and frontal cortices, respectively. Another study conducted to assess the possible role of apoptosis in autism found that Western blotting quantification of *Bcl-2* presented a significant 34– 51% decrease in autistic cerebellum [14, 15]. In this study, it was found that *RALB* was downregulated in 31.5% of autistic cases.

Considering the heterogeneity of ASD symptoms, severity, etiology, and neural signatures the divergences in the *RALB* expression levels in this study could be a result of the complex interaction of various genetic and environmental factors. Therefore, these expression results could be explained with comparison to the methylation pattern of the target *RALB* gene.

MethyLight assay result demonstrated no methylation in the selected area for both control and patient samples. Considering that MethyLight assay is a methylation-specific PCR method and accordingly it is restricted only to the CpGs that are located among the region that the probe is designed to bind to.

In this study, the dysregulation of *RALB* between autistics and control individuals indicated that 57.9% of the cases were upregulated, while 31.5% were downregulated and 10.% showed a normal level in the expression of the *RALB gene*.

As a consequence, the results of qRT-PCR illustrated divergences in the expression levels in autistic cases. Also, to make sure that the differences in the expression levels do not indicate genetic mutation involvement, the *RALB* gene was sequenced in all the autistic samples that showed upregulation. Except for only one sample, all the other 10 samples did not elucidate any mutation. Recently, several studies have reported a high expression level of *RALB* in various tumors, including pancreatic, prostate, bladder, and lung cancers. In these studies, the over-expression of *RALB* was found *to* contribute to tumorigenesis by regulating cell migration, which is a required process of tumor invasion and metastasis [16]. Furthermore, several ASD neural hallmarks strongly related to the abnormalities of cell migration including focal cortical dysplasia (FCD), minicolumnar abnormalities, variable laminar patterns, heterotopias, and alterations in neuronal density [17]. Since the *RALB* gene is considered an essential regulator of cellular migration processes, it is most likely that a high expression *RALB* influences the brain functioning and progression of disorders in autistic patients. In this study, it was found that *RALB* was highly expressed in 57.9 % of autistic cases.

However, highly-expressed *RALB* has been also found to be implicated in many other oncogenic activities such as regulation of vesicle trafficking, and enhancement of gene transcription. Moreover, *in vitro* studies have shown that downregulation of *RALB* increase drug sensitivity in carcinomas [18, 19]. Another recent study demonstrated that downregulation of *RALB* stimulates apoptosis in leukemia cells through de-phosphorylation of *TBK1*, and subsequently decrease the expression level of *NF-κB,* which affects pro-survival genes such as *Bcl-2* [20]. There is a possibility that this pathway dysregulation occurs in autism neural disorder as well.

CONCLUSION

This study was set out to investigate the hypothesis that variation of *RALB* expression levels in ASD patients could be associated with differences in the *RALB* methylation level. This study found considerable dysregulation of *RALB* expression levels.

The present study indicated that *RALB* expression has differential levels in autistic patients. Furthermore, there was no difference in *RALB* methylation level in the candidate promoter region in autistic and healthy samples. Regardless of the negative results of methylation analysis, the dysregulation in the expression of *RALB* could clarify and point to its function in ASD. The current study only

examined the methylation patterns in specific CpG regions, therefore, it seems that further experimental analyses are needed to estimate the methylation level in a wide-range and determine specific biomarker of ASD in the Saudi population.

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Conflict of interest: None

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REFERENCES

[1] Simanshu DK, Nissley DV, McCormick F. RAS proteins and their regulators in human disease. Cell. 2017;170(1):17-33.
[2] Shirakawa R, Horiuchi H. Ral GTPases: crucial mediators of exocytosis and tumourigenesis. J Biochem. 2015;157(5):285-99.
[3] Lalli G, Hall A. Ral GTPases regulate neurite branching through GAP-43 and the exocyst complex. J Cell Biol. 2005;171(5):857-69.
[4] Jullien-Flores V, Mahé Y, Mirey G, Leprince C, Meunier-Bisceuil B, Sorkin A, et al. RLIP76, an effector of the GTPase Ral, interacts with the AP2 complex: involvement of the Ral pathway in receptor endocytosis. J Cell Sci. 2000;113(16):2837-44.
[5] Wen Y, Herbert MR. Connecting the dots: overlaps between autism and cancer suggest possible common mechanisms regarding signaling pathways related to metabolic alterations. Med Hypotheses. 2017;103:118-23.
[6] Modabbernia A, Velthorst E, Reichenberg A. Environmental risk factors for autism: an evidence-based review of systematic reviews and meta-analyses. Mol Autism. 2017;8(1):1-6.
[7] Hassan TR, Mohammed AS, El Desouky MA, Ibrahim MA, Elhefny MA. Evaluation of cellular tumor suppressor protein p53 antigen and 5-Methylcytosine (methylated DNA) as a Diagnostic Biomarker for Hepatocellular Carcinoma in Egypt. J Biochem Tech. 2019;10(3):98-104.
[8] Ladd-Acosta C, Hansen KD, Briem E, Fallin MD, Kaufmann WE, Feinberg AP. Common DNA methylation alterations in multiple brain regions in autism. Mol Psychiatry. 2014;19(8):862-71.
[9] Zhubi A, Chen Y, Guidotti A, Grayson DR. Epigenetic regulation of RELN and GAD1 in the frontal cortex (FC) of autism spectrum disorder (ASD) subjects. Int J Dev Neurosci. 2017;62:63-72.
[10] Nguyen A, Rauch TA, Pfeifer GP, Hu VW. Global methylation profiling of lymphoblastoid cell lines reveals epigenetic contributions to autism spectrum disorders and a novel autism candidate gene, RORA, whose protein product is reduced in autistic brain. FASEB J. 2010;24(8):3036-51.
[11] Truong PK, Lao TD, Le TA. CDKN2A Methylation-an epigentic biomarker for cervical cancer risk: A meta-analysis. Pharmacophore. 2020;11(2):21-9.
[12] Constantino JN, Marrus N. The early origins of autism. Child Adolesc Psychiatr Clin. 2017;26(3):555-70.
[13] Schaaf CP, Zoghbi HY. Solving the autism puzzle a few pieces at a time. Neuron. 2011;70(5):806-8.
[14] Sasaki T, Kitagawa K, Yagita Y, Sugiura S, Omura-Matsuoka E, Tanaka S, et al. Bcl2 enhances survival of newborn neurons in the normal and ischemic hippocampus. Neurosci Res. 2006;84(6):1187-96.
[15] Araghi-Niknam M, Fatemi SH. Levels of Bcl-2 and P53 are altered in superior frontal and cerebellar cortices of autistic subjects. Cell Mol Neurobiol. 2003;23(6):945-52.
[16] Khawaja H, Campbell A, Roberts JZ, Javadi A, O'Reilly P, McArt D, et al. RALB GTPase: a critical regulator of DR5 expression and TRAIL sensitivity in KRAS mutant colorectal cancer. Cell Death Dis. 2020;11(10):1-8.
[17] Wegiel J, Kuchna I, Nowicki K, Imaki H, Wegiel J, Marchi E, et al. The neuropathology of autism: defects of neurogenesis and neuronal migration, and dysplastic changes. Acta Neuropathol. 2010;119(6):755-70.
[18] Seguin L, Kato S, Franovic A, Camargo MF, Lesperance J, Elliott KC, et al. An integrin β 3–KRAS–RalB complex drives tumour stemness and resistance to EGFR inhibition. Nat Cell Biol. 2014;16(5):457-68.
[19] Khawaja H, Campbell A, Roberts JZ, Javadi A, O'Reilly P, McArt D, et al. RALB GTPase: a critical regulator of DR5 expression and TRAIL sensitivity in KRAS mutant colorectal cancer. Cell Death Dis. 2020;11(10):1-8.
[20] Eckfeldt CE, Pomeroy EJ, Lee RD, Hazen KS, Lee LA, Moriarity BS, et al. RALB provides critical survival signals downstream of Ras in acute myeloid leukemia. Oncotarget. 2016;7(40):65147.