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The correlation between copy number variation in Chromosome 14 and DNA methylation in Saudi autistic children

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Abstract. – OBJECTIVE: Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder that represents a range of aberrant behaviour symptoms such as repetitive behaviours and defects in social communication. The prevalence of ASD has been increasing worldwide and many studies have reported that both genetic and epigenetic factors play an important role in the etiology of this disorder. The aim of this study was to investigate the implementation of DNA methylation and Copy number variation (CNV) in the diagnosis of ASD.

PATIENTS AND METHODS: This study was carried out on 14 Saudi autistic children and four of their healthy siblings. Comparative genomic hybridization array was used to identify CNV in chromosome 14 and MethyLight qPCR was used to estimate levels of DNA methylation.

RESULTS: The results identified CNVs in six cytobands in chromosome 14 for 13 out of 14 autistic samples: 14q11.1-q11.2, 14q11.2, 14q12, 14q21.1, 14q32.2, and 14q32.33. However, some of these cytobands were also found in normal samples with different sizes. Interestingly, chromosomal abnormalities in 14q11.1-q11.2 was only found in ASD samples. The result also showed an increase in methylation ratio of ASD samples in those CNV regions compared with their siblings.

CONCLUSIONS: The findings suggest that CNV in 14q11.1-q11.2 might be a potential target in ASD diagnosis and further work is required to detect which biological pathways are affected.

Key Words:
Autism, ASD, Neurodevelopmental disorder, CNV.

Abbreviations
Autism Spectrum Disorder; aCGH: Array comparative genomic hybridization; CNV: Copy number variation; WHO: World Health Organization; LINE-1: long interspersed nucleotide elements; DSM-5: Diagnostic and Statistical Manual of Mental Disorders version 5, CpGI: CpG island, COL1A1: Collagen Type I Alpha 1 Chain; OR: Olfactory receptor. PMR: Percentage of methylated ratio.

Introduction

Autism

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder where impairments in social communication and repetitive behavior are dominant features of this disorder[1]. ASD is a heterogeneous disorder that is caused by several factors, which lead to variable phenotypes amongst autism cases.

According to the World Health Organization (WHO), 1 in 100 children is diagnosed with ASD around the world[2]. In fact, the prevalence of ASD in Saudi Arabia is anonymous, because of less public awareness of this disorder or the absence of documented national statistical reports that count the number of autistic children in schools and health centers in all regions in the country. However, a previous study in the capital city of Saudi Arabia reported the prevalence of ASD among Saudi children between two and four years old was estimated to be 1:40, 25 per 1000, with a ratio of 3:1 for male to female[3].

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Copy Number Variation

Copy number variation (CNV) refers to a phenomenon in which the number of repeats of a specific segment of DNA varies among different individuals' genomes[4]. CNVs arise from unbalanced rearrangements of genes by either amplification or deletion, which may directly or indirectly affect the expression of several genes. CNV plays a critical role in a disease phenotype. CNV is also recognized as an important class of risk factors for several psychiatric disorders. Furthermore, there are several CNV regions which are associated with autistic cases such as 1q24.2, 2q37.3, 3p26.2, 4q34.2, 6q24.3, 7q35, 13q13.2-q22, 15q11-q13, 15q22, 16p11.2, 17p11.2, 22q11, 2q13, and Xp22[5].

DNA Methylation

DNA methylation is one of the most well-known epigenetic silencing mechanisms, which is described as a covalent attachment of the methyl group to cytosine residues in DNA sequence[6]. DNA methylation is associated with several neurological disorders including ASD. The severity of the autistic phenotypes could be related to aberrant DNA methylation level at a specific site across the genome[7]. Moreover, a previous study in monozygotic twins found significantly different methylation in many regions associated with ASD[8].

Correlation Between CNV and DNA Methylation

Although genetic and epigenetic mechanisms are associated, the correlation between CNV and DNA methylome is unclear. To date, few studies have investigated this relation. A study that examined the association between structural rearrangements of the CNV and germline DNA methylation analyzed 400 human sperm samples using array comparative genomic hybridization (aCGH) and reported that 23% of structural rearrangements are associated with hypomethylation[9].

In cancer cells, it has been reported that amplification and deletion in CNV cause a change in DNA methylation around CpG islands and the CpG ocean due to the redistribution of the methylase complex in a comparatively small region of the genome[10].

However, long interspersed nucleotide elements (*LINE-1*) and Alu repeats transposable elements, where their methylation represents distinct measures of methylation in different parts of the whole genome, play a role in the formation of CNVs[11,12]. Additionally, the activity of transposable elements is well-known to be suppressed by CpG methylation[13,14] thus DNA methylation could play a critical role in CNV formation and genome stability. There are limited studies on the correlation between CNV and DNA methylation. Therefore, this study aimed to investigate the correlation of DNA methylation and CNV in ASD.

Patients and Methods

Study Population

Saudi autistic children and their normal siblings aged between 3 and 12 years old were included in this study. The children were diagnosed based on the Diagnostic and Statistical Manual of Mental Disorders version 5 (DSM-5)[15], the characteristics of ASD children are presented in Table I. The autistic patients were 14 children including twelve males and two females, and the normal (control) samples were four children including three males and one female. The children's parents signed consent forms for the agreement of the participation of their children in this study.

Genomic DNA Extraction

Peripheral blood from children was collected into EDTA anticoagulant tubes at the autistic clinic in Jeddah to extract DNA. Genomic DNA was extracted from 2 ml of whole blood samples using the QIAamp DNA Mini Kit as instructed by the manufacturer (QIAamp DNA mini® Kit, Qiagen, Hilden, Germany).

Array Comparative Genomic Hybridization (aCGH)

For detection of CNV in samples, DNA samples were analyzed by aCGH using a SurePrint G3 Human CGH Microarray 4x180K (Agilent, Santa Clara, CA, USA). The terms amplification, gain, loss and deletion depend on the mean log ratio, where the normal range is between 0 and +0.25, and 0 and - 0.25. However, the size of CNV amplification is greater than +0.60. While in the gain case, the size of CNV ranged from +0.25 to +0.58. On the other hand, CNV deletion is greater in size than -1.0. While in the loss case, the CNV size ranged between -0.25 and -0.99.

Bisulfite Conversion

The ability to detect and measure DNA methylation using the MethyLight qPCR technology requires converting the initial template such that

Table I. Characteristics of ASD children.

Patient numer	Gender	Age (Years)	Birth type	Severity of ASD	Onset of symptoms	Parental consanguinity	Family history of autism
Autistic Sample-1	Male	12	Caesarean	Simple/level1	after 2 years	No	His cousin
Autistic Sample-2	Female	9	SVD*	Mild/level2	after 2 years	No	No
Autistic Sample-3	Male	10	SVD	Simple/level1	after 2 year	No	No
Autistic Sample-4	Male	8	Caesarean	Mild/level2	after 2 years	No	No
Autistic Sample-5	Male	8	Caesarean	Mild/level2	after 6 months	cousin	No
Autistic Sample-6	Male	6	Caesarean	Mild/level2	after 2 years	No	Yes
Autistic Sample-7	Male	7	Caesarean	Simple/level1	after 2 year	cousin	His cousin
Autistic Sample-8	Male	12	SVD	Mild/level2	after 2 years	No	No
Autistic Sample-9	Male	8	Caesarean	Sever/level3	after 2 years	No	His brother
Autistic Sample-10	Male	7	SVD	Mild/level2	after 1 year	cousin	His cousin
Autistic Sample-11	Male	10	Caesarean	Mild/level2	after 2 years	No	No
Autistic Sample-12	Female	10	SVD	simple/level1	after 2 years	No	Her brother
Autistic Sample-13	Male	9	Caesarean	Mild/level2	after 2 years	No	No
Autistic Sample-14	Male	8	Caesarean	Mild/level2	after 2 years	No	No

* SVD: Spontaneous Vaginal Delivery.

methylated cytosines can be differentiated from unmethylated cytosines. Unmethylated cytosines in DNA strand were converted to uracil through sodium bisulfite treatment using EpiTect Bisulfite Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Methyl Light qPCR

MethyLight primers and probes were designed using the MethPrimer website (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) for two CpG islands (CpGIs) within candidate CNV regions as well as Collagen Type I Alpha 1 Chain gene (*COL1A1*) which was utilized as an endogenous control for the MethyLight qPCR assay.

Primers and probes for methylation analysis of two CpGIs and *COL1A1* were as follows: for CpGI-1 probe (FAM 5'-CGT TTATTAGGTAGCGGCGT-3' BHQ1), and CpGI-2 probe (FAM 5'-CGTCGCGTTTTT TAATTTAGACG-3' BHQ1).

The amplification oligonucleotide primers, CpGI-1F (5'-GATTTGGAGAAGTATTTG GTTTTT-3'), CpGI-1R (5'-ACCCAAAAT CTACTTCCCAAAC-3'), CpGI-2F (5'-GT GAATTATAGTAGTTGTGGTAGT-3'), and for CpGI-2R (5'-ACTACAAATTTC CTCCCTCTAAC-3'). For the internal control and normalization, *COL1A1* probe (HEX5'-CCTTCATTCTAACCCAATACC TATCCCACCTCTAAA-3'BHQ) targeting the methylation independent and bisulfite-conversion-dependent sequence, was used with the amplification oligonucleotide primers, *COL1A1*-F (5'-TCTAACAATTAT AAACTCCAACCAC-CAA-3') and *COL1A1* -R (5'-GGGAAGATG-GGATAGAAGGGA ATAT-3').

The methylation level was analyzed using EpiTect MethyLight PCR Kit (Qiagen, Hilden, Germany), 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 dinucleotide among ASD patients. The reactions were carried out using the StepOne Plus Real Time PCR machine (Applied Biosystem, Foster City, CA, USA).

Results

CNVs Analysis

In this study, array comparative genomic hybridization results have shown CNVs in several regions in chromosome 14 for fourteen autistic children and four control samples. However, the results identified CNV on six cytobands of chromosome 14 for 13 out of 14 autistic samples and some of these cytobands were found in normal samples with different sizes. These six cytobands are: 14q11.1-q11.2, 14q11.2, 14q12, 14q21.1, 14q32.2, and 14q32.33.

The aCGH experimental results of the autistic and normal samples are shown in Table II and Table III, respectively. The aCGH analysis revealed CNVs on cytoband 14q11.1 -q11.2 (Figure 1), which existed in 3 out of 14 autistic samples but were not found in normal samples. This cytoband was lost in two

Table II. The aCGH experiment result of the autistic and normal samples.

Sample	Gender	Cytoband	Start	End	CNV type	Size (kb)
Autistic sample – 1	M	14q11.1 - q11.2	18446762	19491517	Loss	1044.756
		14q11.2	21368989	22131455		762.467
		14q32.33	105476748	106285938	Amplification	809.191
			106205848	106239795	Gain	33.948
			105476748	105874352	Amplification	397.605
Autistic sample – 2	F	14q11.1 - q11.2	18446762	19491517	Amplification	1044.756
		14q11.2	21457258	22131455	Loss	674.198
		14q32.33	105476748	106028995	Amplification	552.248
			105476748	105609525		132.778
Autistic sample – 3	M	14q11.2	21457258	22131455	Loss	674.198
		14q32.33	105476748	106285938	Amplification	809.191
			105476748	105874352		397.605
			105476748	105584067		107.32
Autistic sample – 4	M	14q11.1 - q11.2	18919591	19491517	Loss	571.927
		14q11.2	21389103	22131455	Loss	742.353
		14q21.1	40686163	40726989	Loss	40.827
		14q32.33	105476748	105874352	Amplification	397.605
			105584008	105632227		48.22
			105923159	106285938		362.78
Autistic sample – 5	M	14q11.2	21617709	22101647	Loss	483.939
		14q32.33	105476748	106028995	Amplification	552.248
			106061322	106285938		224.617
Autistic sample – 6	M	14q11.2	22387418	23011311	Loss	623.894
		14q32.33	106405703	106803307	Amplification	397.605
			106852114	107214893	Gain	362.78
Autistic sample - 7	M	14q11.2	22139067	23061615	Loss	922.549
		14q32.2	101291239	101292466	Gain	1.208
		14q32.33	106405703	106803307	Amplification	397.605
			106852114	107214893	Gain	362,78
Autistic sample – 8	M	14q32.33	106906901	107141075	Amplification	234.175
			106405703	106803307		397.605
			106405703	107214893		809.191
Autistic sample – 9	M	14q11.2	22368864	22952279	Loss	583.416
		14q32.33	106405703	107214893	Amplification	809.191
			106636701	106750038		113.338
			106405703	106513022		107.32
Autistic sample - 10	M	14q32.2	101289600	101298326	Amplification	8.727
		14q32.33	106405703	106803307		397.605
			106852114	107214893		362.78
Autistic sample - 11	M	14q32.33	106538421	106803307	Deletion	264.887
Autistic sample - 12	F	-----	----	----	----	----
Autistic sample - 13	M	14q11.2	19434575	20439064	Gain	1004.49
Autistic sample - 14	M	14q11.2	20229107	20421677	Gain	192.571
		14q12	29236277	29237889	Loss	1.613
		14q32.33	107148739	107182658		33.92
Normal sample - 1	M	14q11.2	21438704	21966929	Loss	528.226
		14q32.33	105476748	106280474	Amplification	803.727
		14q32.33	106205848	106253803	Gain	47.856
Normal sample - 2	M	14q11.2	19376762	20421677	Loss	1044.916
		14q21.1	41616413	41657239	Deletion	40.827
		14q32.33	106405703	106803307	Amplification	397.605
		14q32.33	106852114	107214893	Gain	362.78
Normal sample- 3	F	14q21.1	42918529	43135217	Loss	216.689
		14q32.33	107141016	107182658	Loss	41.643
Normal sample- 4	M	14q32.2	101291158	101293776	Gain	2.619

Table III. List of genes on CNV regions.

Cytoband	Genes
14q11.1 - q11.2	*OR11H12, LINC02297, POTEG, LOC101929572, DUXAP10, LINC01296, BMS1P18, BMS1P17, BMS1P22, POTEM, LOC100508046, OR11H2, OR4Q3, OR4M1, OR4N2, OR4K3, OR4K2, OR4K5, OR4K1, DUXAP9,LINC02297*
14q11.2	*LOC105370401, LINC02332, DAD1, SLC7A7, CDH8*
14q32.2	*MEG3*
14q32.33	*ADAM6, LINC00226, LINC00221, MTA1, PACS2, TEDC1, ELK2AP, MIR8071-2, TMEM121, CRIP2, GPR132, MIR5195*

patients (male) and gained in one female patient. The other lost cytoband was reported on 14q11.2 (Figure 1) which was in seven autistic children (male) and one female as well as two normal samples (male) but varying in size, approximately from ~483 Kb to ~1044 Kb. On the other hand, the cytoband 14q11.2 gained CNV in two autistic patients (male) with different sizes of 192 Kb and 1004 Kb (Figure 1). Moreover, the lost region of 1.613Kb on 14q12 in one autistic child was detected (Figure 2). In addition, the lost region of 40.827 Kb on 14q21.1 in one autistic child (male) and two normal samples was detected (Figure 3). Also, the amplification and gain on 14q32.2 were observed in two autistic children (male) and one normal sample (Figure 4). Moreover, the amplification region on 14q32.33 in ten autistic children (male/female) and two normal samples was detected, whereas one autistic child had deletion and one normal sample had a loss in the same region (Figure 5).

Furthermore, a group of candidate genes was found in CNV regions in chromosome 14 as listed in Table IV. However, CNVs in 14q11.1-11.2 affect many critical genes including *SLC7A7*, three of *POTE* gene family as well as two genes of *DUXA* homeobox gene family, several genes of Ribosome Biogenesis Protein *BMS1* Homolog Pseudogene, *DAD1* gene, nine olfactory receptor genes, and *CHD8* gene.

DNA Methylation Analysis

MethyLight qPCR was used in this study to determine the relative prevalence of a particular pattern of methylated CpG dinucleotides in two CpGIs within the region of CNVs in cytoband 14q11.1-q11.2 that were found in three autistic samples comparing to the methylation level of two normal samples. The CpGIs were identified using the UCSC Genome Browser, 16 CpG islands were found in CNV in cytoband 14q11.1-q11.2 (chr14:18446762-19491517), where the size of CNV was 1,044,756 bp (Figure 6). Two CpGIs were examined, the first CpGI, which was investigated in this study, was located in (chr14:19026535-19027143), the probe indicated three CpGs in (chr14:19026803-19027100). The second CpGI was located in (chr14:19344831-19345388), the probe indicated four CpGs in (chr14:19345169-19345192). The percentage of methylated ratio (PMR) of CpGIs for all samples was determined as shown in Table IV and V. The PMR of CpGI-1 was above 40% in two autistic samples, but no signal was detected for the third autistic sample. However, the two normal sam-

Table IV. The PMR of CpGI-1.

Sample		Quantity of collagen	Quantity of CpG-1	PMR
Methylated DNA	control	0.36883768	0.36130403	100%
Autistic sample - 1	ASD	0.41559919	0.16547464	40.6%
Autistic sample - 2	ASD	0.35846217	0.15895804	45.3%
Autistic sample - 4	ASD	0.14889914	804.092358	0.0%
Normal sample - 1	Normal	0.2543581	0.05188924	20.8%
Normal sample - 2	Normal	0.28347832	0.07671731	27.6%

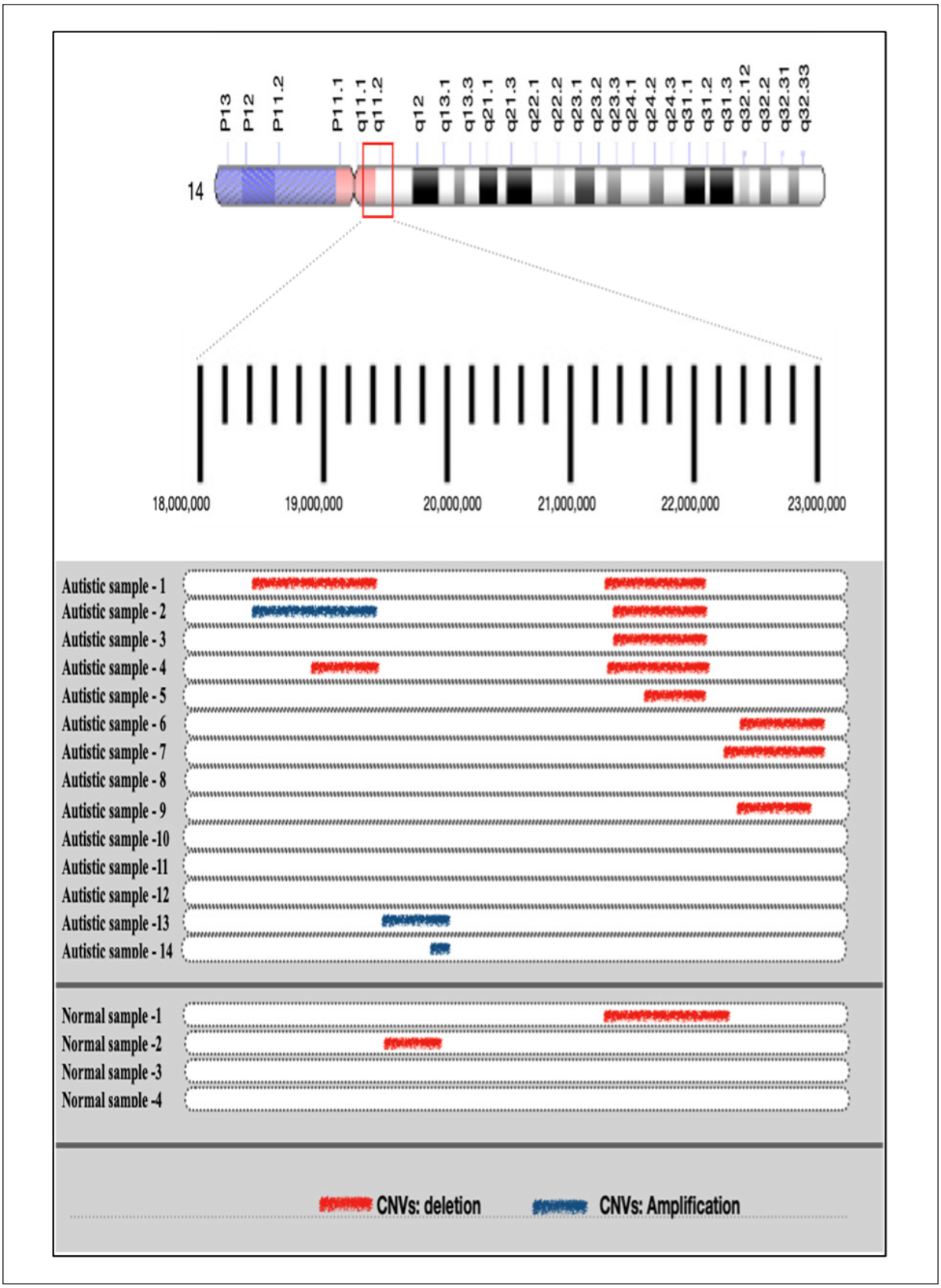

Figure 1. CNVs in cytoband 14q11.1-q11.2 and 14q11.2 in autistic and normal samples.

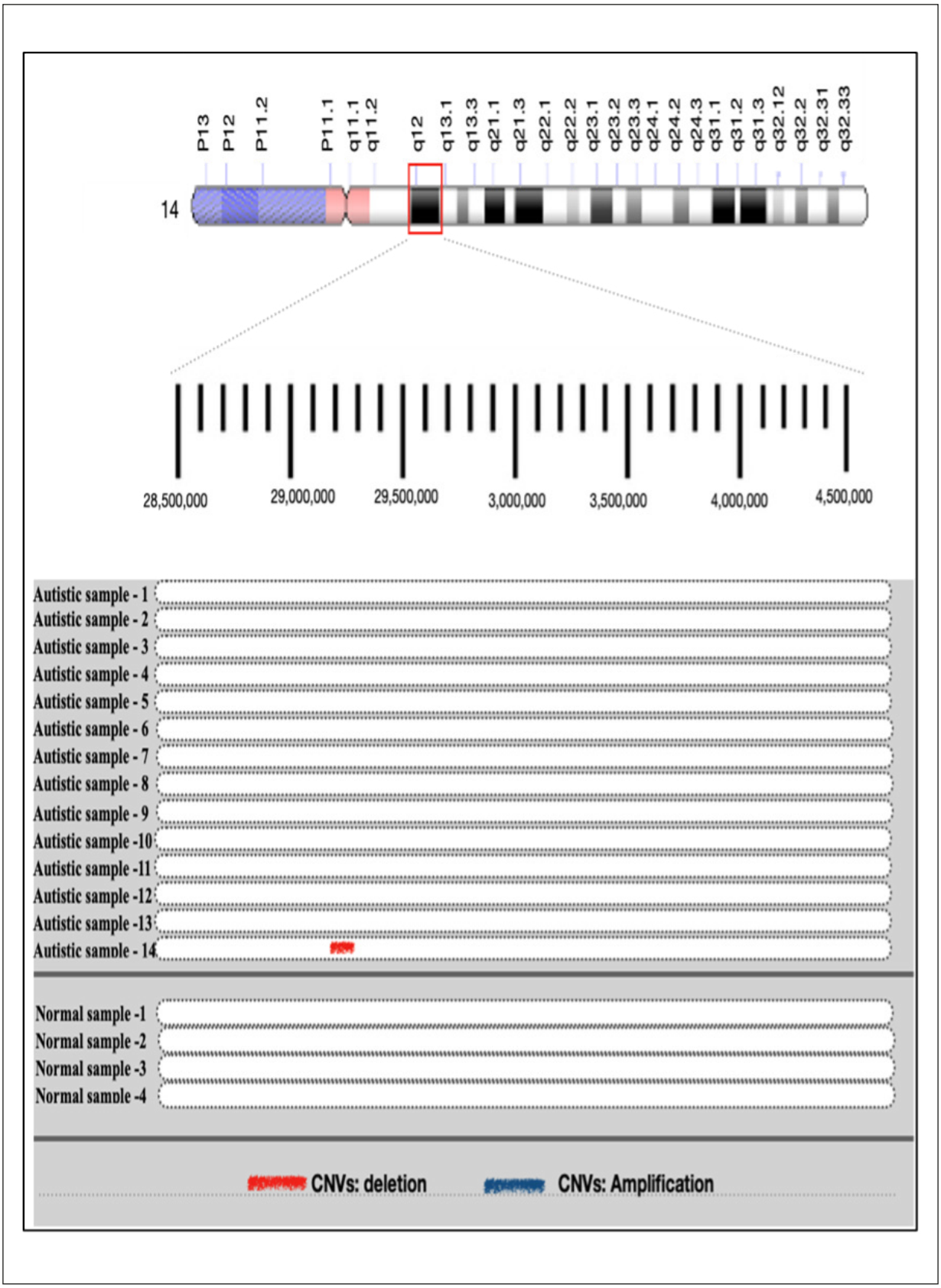

Figure 2. CNVs in cytoband 14q12 in ASD.

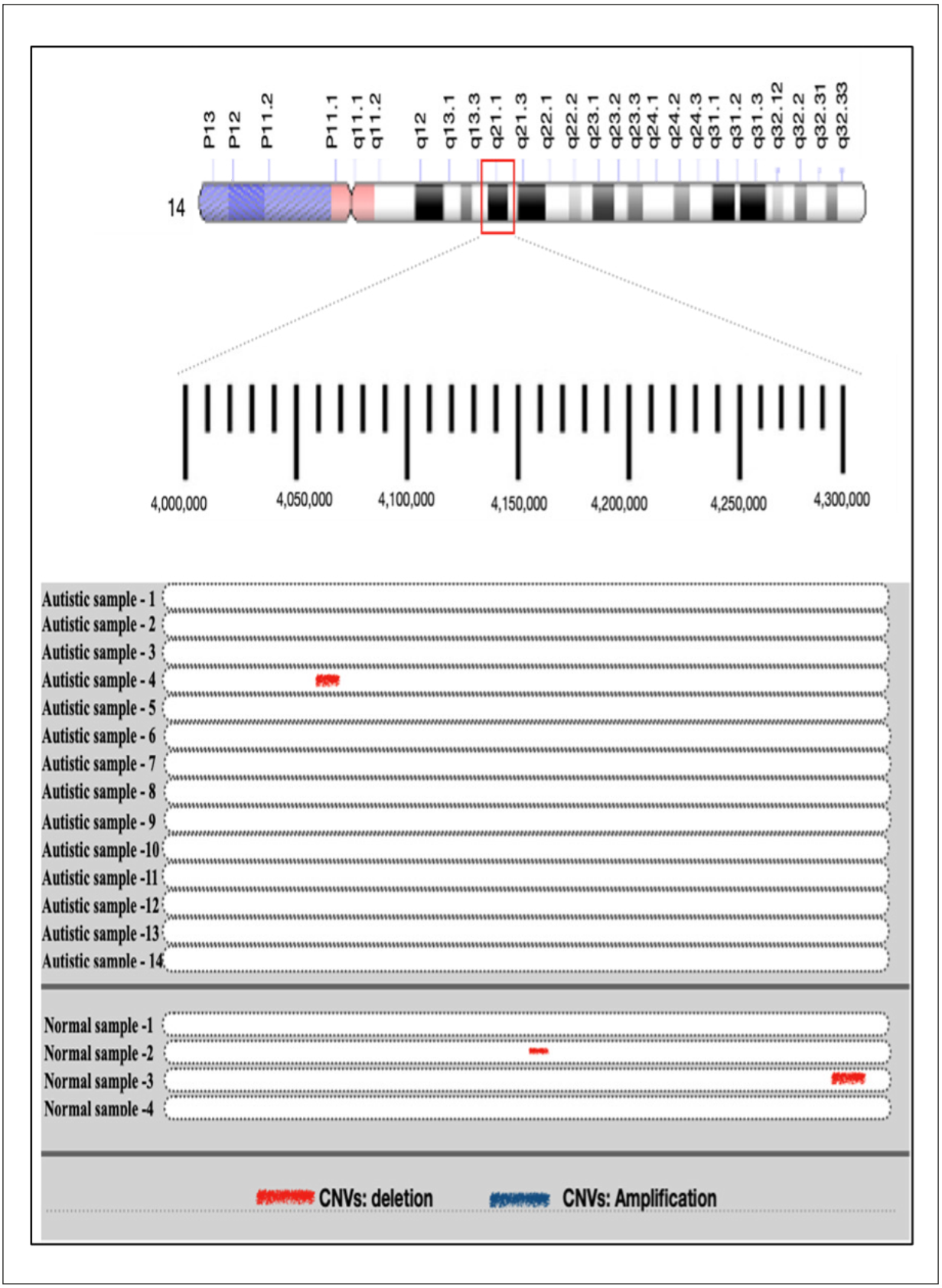

Figure 3. CNVs in cytoband 14q21.1 in ASD samples comparing to normal samples.

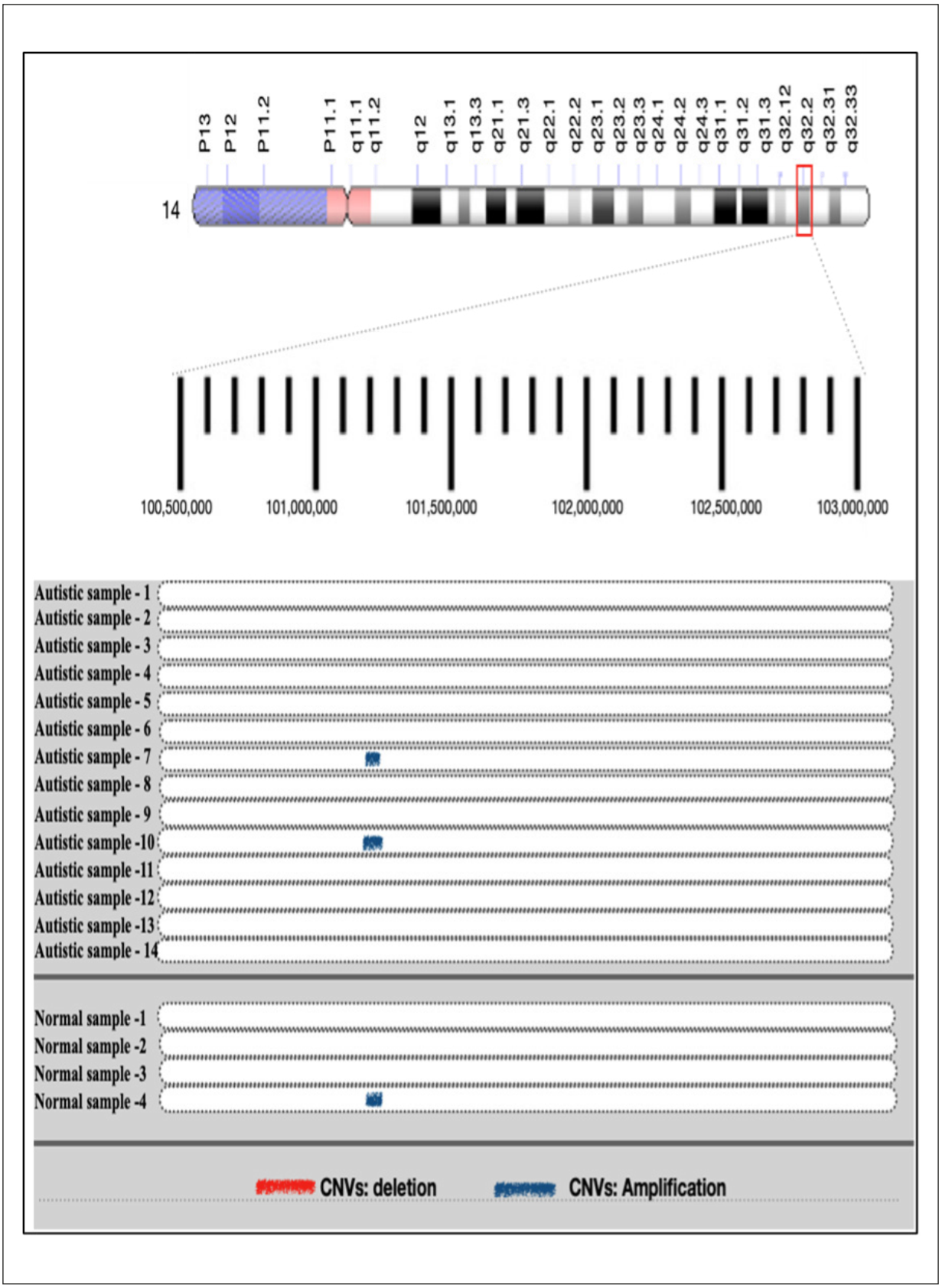

Figure 4. CNVs in cytoband 14q32.2 in ASD samples comparing to normal samples.

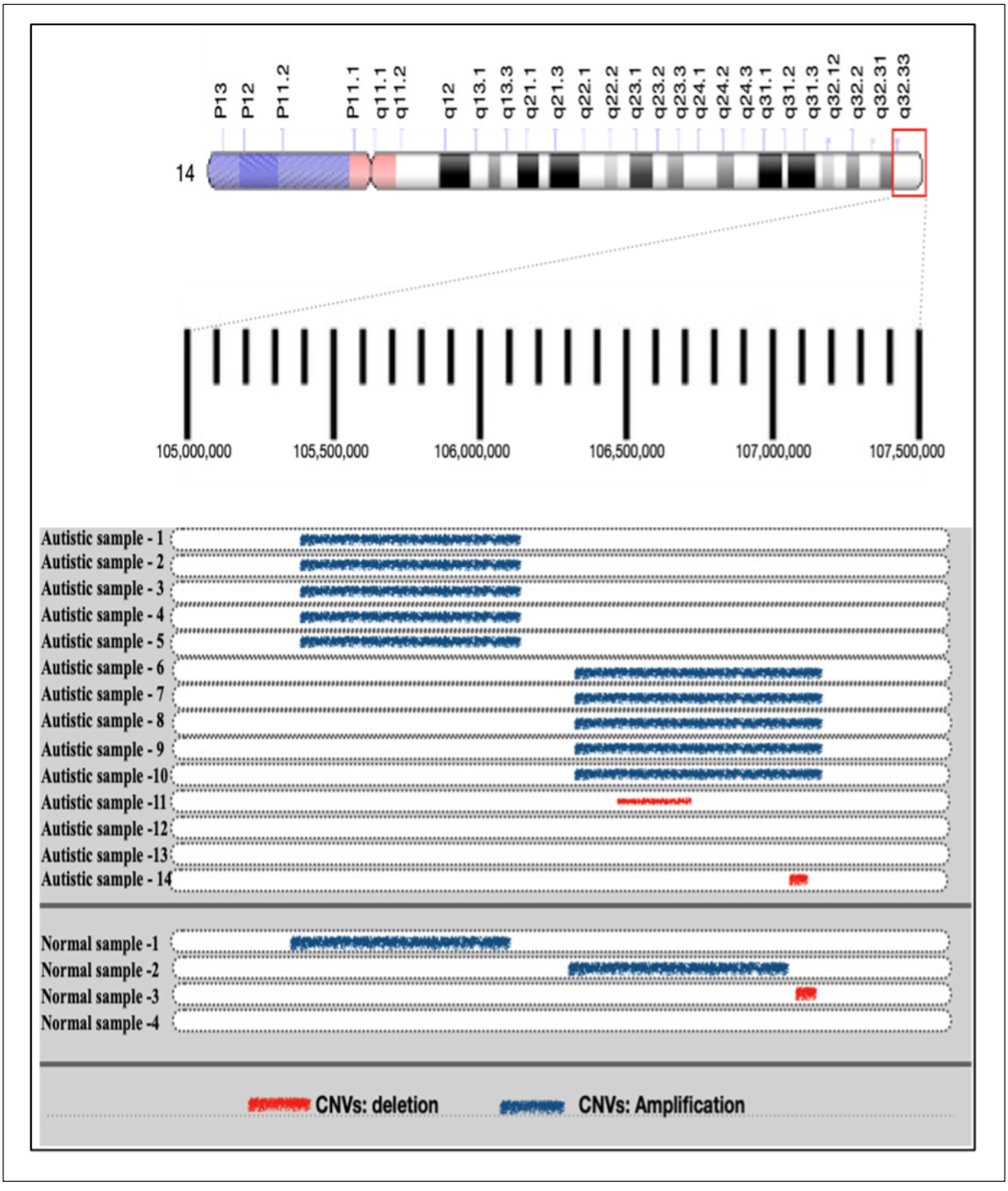

Figure 5. CNVs in cytoband 14q32.33 in ASD samples comparing to normal samples.

ples showed methylation ratios below 28% (Figure 7). The PMR of CpGI-2 at 36.6%, 65.5%, and 82.3% were observed in autistic samples 1, 2 and 4, respectively. However, the two normal samples showed methylation ratios below 63% (Figure 8). PMR value of 10 or higher is considered evidence

for methylation whereas samples with PMR of less than ten are considered unmethylated[16].

Discussion

Studying genetic and epigenetic differences among ASD children is a powerful tool that could help in assessing the biological mechanism and therapeutic targets for ASD. Our findings support the hypothesis that CNV in Chromosome 14 may be associated with ASD pathogenesis. The results identified CNV in chromosome 14 for 13 out of 14 autistic samples (93%) while the previous study by our research group indicated 6 out of 15 autistic samples (40%) have multiple CNVs in Chromosome 22, including significant genes such as *TBX1, LIF, SEPT5, GNB1L, YWHAH, SHANK3, PPARA, SYN3, TUG1, MLC1, PANX2*, and *HDAC10*[17], which suggested that genes at CNVs in chromosome 14 could be more significant in contributing to etiology of ASD.

This study found three genes belonging to a *POTE* gene family in CNV in three autistic samples (*POTEG, POTEH-AS,* and *POTEM). POTE* genes play a critical role in apoptosis and cytoskeletal functions and are therapeutic targets and biomarkers for several types of cancer[18,19]. Several studies found mutations in genes belonging to POTE family genes in ASD cases. A previous study found a mutation in *POTED* gene in autistic children[20]. Another study demonstrated that the aberration in *POTEKP* could lead to defects in cell-to-cell communication between neurons in ASD[21].

DUXAP10 and *DUXAP9* are pseudogenes that encode DNA-binding proteins, and several researchers found that these genes play roles in early embryonic development in addition to promoting proliferation, migration, and apoptosis inhibition[22,23]. A previous study reported that *DUXAP10* could interact with Histone Demethylase Lysine Specific Demethylase1, which regulates histone methylation[24]. The previous finding for mutation in *DUXAP10* pointed out that it might be contributing to the alteration of epigenetic events.

SLC7A7 gene codes for a protein that is involved in transporting a certain building block of protein, namely lysine, arginine, and ornithine. The association between *SLC7A7* and ASD has been reported in several studies[25-28]. Olfactory receptor (*OR*) genes code for proteins involved in the smell sensory function. The *OR* family roughly consists of 900 members[29,30] of large families that coordinate in clusters in human[31]. The *OR* clusters in chromosome 14q11.2 are considered the most CNV-enriched region. The CNV profile of the *OR* cluster on chromosome 14q11.2 has shown sex bias which significantly affects males more than females[30]. The *OR* genes belong to seven-transmembrane G protein-coupled receptor (*GPCR*) superfamily which transmits external physical signals to the inside through G proteins. The *GPCR* has highly conserved regions and is composed of seven transmembrane alpha helices interconnected by three intracellular and three extracellular loops. The olfactory adaptation is an important process that allows the individual to adjust for changing in the environment. Defects in olfaction sensory have been described as predic-

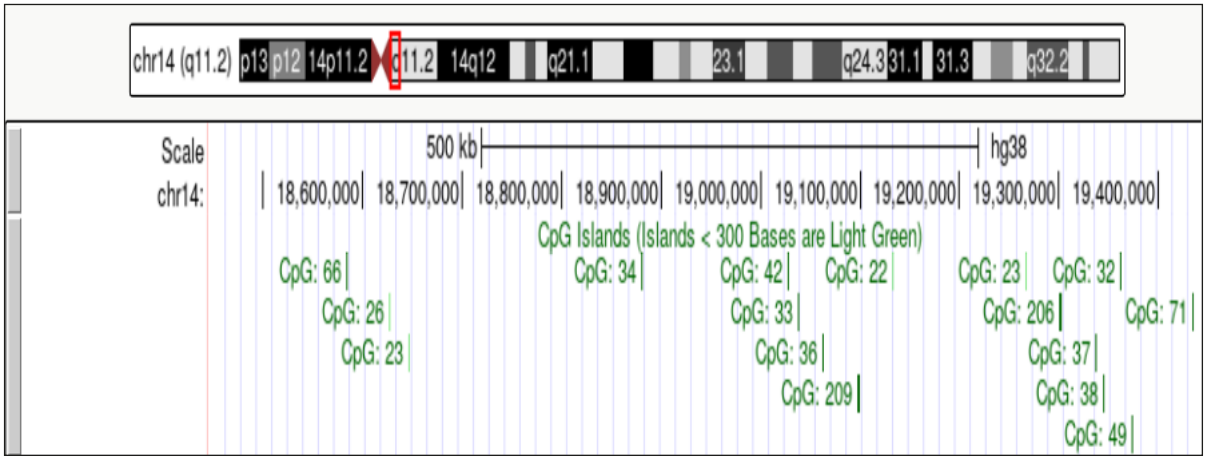

Figure 6. The CpG islands within CNV in cytoband 14q11.1-q11.2.

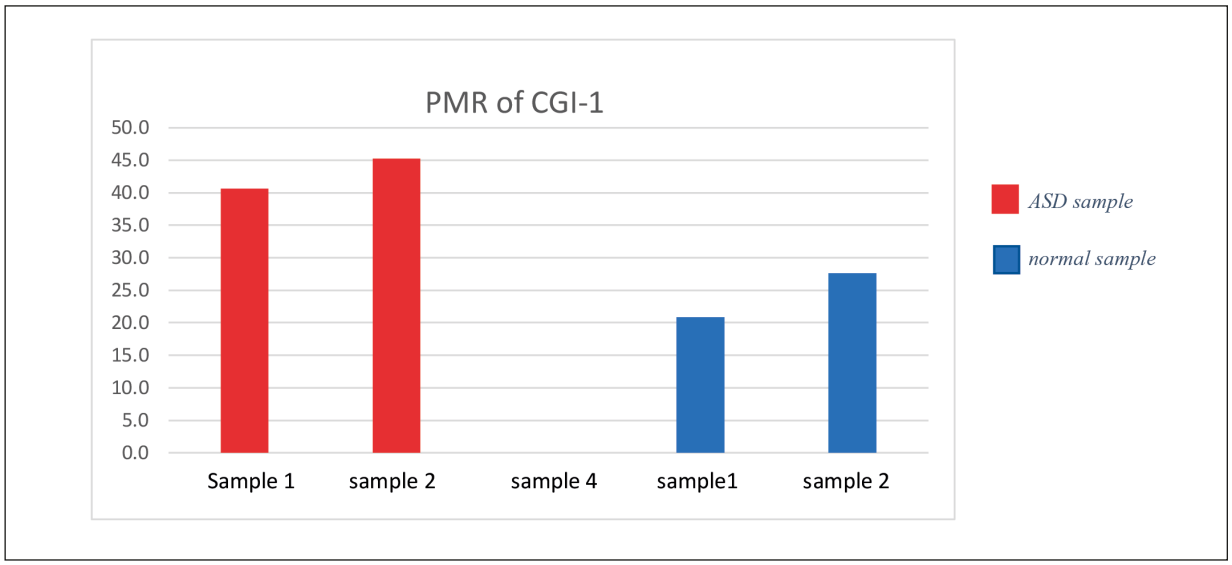

Figure 7. The PMR of CGI-1.

tive causes of social impairment in autistic children[32-36]. A study on the *OR* gene family of 150 individuals for assessing the relationship between CNV and gender demonstrated that CNV among *OR* genes is more significant in males than females, specifically among individuals diagnosed with ASD[37]. Furthermore, a recent study showed that the response of adults with autism to human odors was different from their typical peers. The defect of social interaction and misinterpretation of social emotion among autistic patients could be due to a defect in their ability to sense smell[38].

In 2019, a study examined the olfactory adaptation in nine autistic patients and found less olfactory adaptation in them comparing to normal[36]. Although the *OR* gene family is mainly expressed in olfactory epithelium cell with a functional role in olfaction, some *OR* genes are expressed in a few other cells with poorly understood function. The *OR* genes belong to a large family of G protein-coupled receptors (*GPCRs*) that are located in the cell membrane, which receive an external signal to activate serial interaction that turns adenosine triphosphate (ATP) into cyclic adenosine

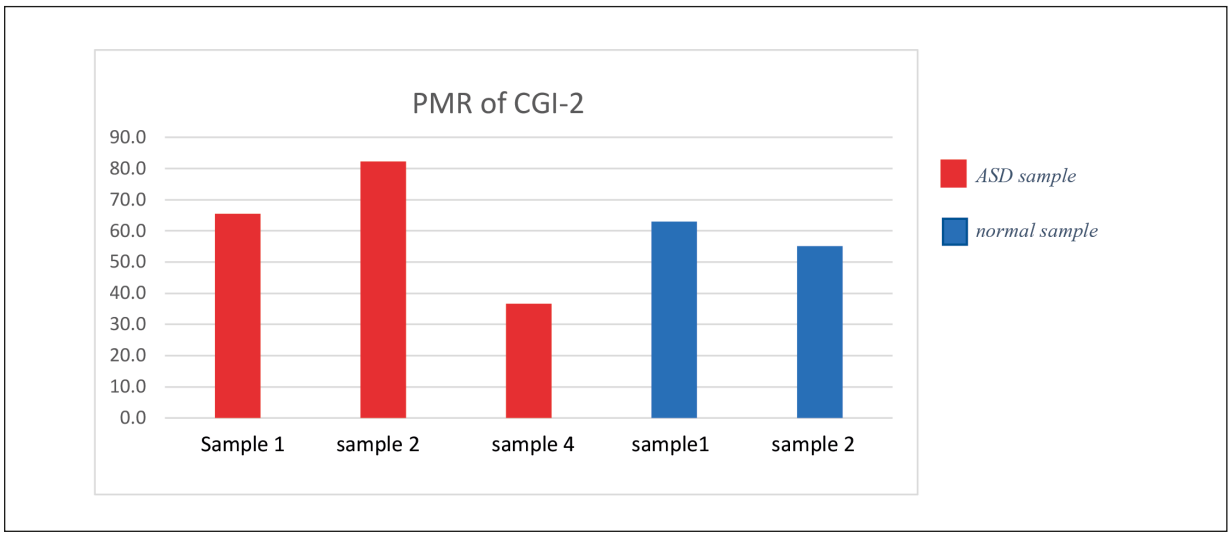

Figure 8. The PMR of CGI-2.

Table V. The PMR of CpGI-2.

Sample		Quantity of collagen	Quantity of CpG-2	PMR
Methylated DNA	control	0.34487045	0.3446615	100%
Autistic sample - 1	ASD	0.40924931	0.26782983	65.5%
Autistic sample - 2	ASD	0.4684885	0.38512473	82.3%
Autistic sample - 4	ASD	0.16051795	0.0587878	36.6%
Normal sample - 1	Normal	0.13808734	0.08696826	63.0%
Normal sample - 2	Normal	0.26108649	0.14363214	55.0%

monophosphate (cAMP) by adenylate cyclase that increases cAMP level in the cell. A previous study proved that cAMP level was increased in olfactory neurons as a consequence of odor stimulation[39]. The increase of secondary messenger cAMP may lead to many activations depending on the cell type and the external signal, one of these activations is increasing the calcium (Ca^{2+}) level. Previous studies have indicated that exaggerated Ca^{2+} signaling plays a vital role in the pathophysiology of autism[40,41]. The aberrant dosage of the *OR* genes in ASD samples could lead to Ca^{2+} imbalance.

DAD1 gene had been linked with cancer[42-44] and ASD where a previous study reported a microdeletion in 14q11.2 with the *DAD1* gene in ASD case[45].

Moreover, Chromodomain Helicase DNA-binding 8 (*CHD8*) is one of the most confident risk factors for ASD. Recently, several studies investigated the role of *CHD8* in the neuron cell[46,47]. One study reported that *CHD8* deficiency prevents dendritic outgrowth and branch development of neurons[48].

This study identified CNV (amplification and gain) on cytoband 14q32.2 for 2 out of 14 autistic children and one normal child. It includes the *MEG3* gene, a maternally expressed imprinted long non-coding RNA located in the Dlk1-Dio3 region. Several studies[49-51] found a mutation in 14q32.2 in autistic patients. However, many studies have investigated the role of *MEG3* in neuro-disorders. One study found 17 miRNAs downregulated in the Dlk1-Dio3 region in the blood sample of schizophrenia patients[52]. Moreover, DNA methylation analysis of intellectually disabled patients found an aberrant DNA methylation in the *MEG3* gene[53]. A previous study indicated that *MEG3* plays a functional role in the dynamic modulation of PTEN/PI3K/AKT signaling during synaptic plasticity in neurons[54]. Recent research performed on 60 rats with Alzheimer's disease indicated that upregulating of *MEG3* enhanced cognitive, decreased neuronal damage, and reduced astrocyte activation in hippocampus tissues in this disease by inhibiting PI3K/Akt signaling pathway[55].

Furthermore, CNV in 14q32.33, including many genes such as *MIR5195* and *MTA1* were reported as ASD candidate genes in many studies[56-58]. *MIR5195* is a gene encoding short non-coding RNAs that are involved in posttranscriptional regulation of gene expression. Several studies of miRNA analyses revealed that *miR-5195-3p* was upregulated in the blood of ASD patients[59] and was highly expressed in febrile seizure patients[60]. However, the overexpression of *miR-5195-3p* was significantly associated with suppression of cell proliferation and induced apoptosis in cancer studies[61-63].

In the second part of this study, Methylghit qPCR was performed to gain insights into the methylation state of two CpGIs in the CNV region. Methylghit is a high throughput semiquantitative methylation analysis based on detecting the fluorescence signals in real-time PCR. The methylation analysis for one autistic sample (female), that displayed amplification in the region of CNV, showed that the methylation ratios in CpGI-1 and CpGI-2 were 45.3% and 82.3%, respectively. The PMR of that sample showed an increase in PMR comparing to other samples. This result is similar to a previous study which identified hypermethylation in amplification regions in 55 cancer patients by using quantitative methylation analysis[64]. However, this finding is contrary to the previous study in which a reduction in DNA methylation near CpGIs in amplification regions was observed[10]. The possible explanation is that Sun et al[10] used data from Illumina 450k array and Illumina 27k array, which are advanced methods compared to the quantitative methods based on real-time PCR that were used in this and Schneider et al[65] studies. Another explanation of the increase in PMR in the amplification region could be attributed to the increase in copy number of that region not to the actual methylation state. Furthermore, the methylation ratio of two autistic samples that

had a loss in CpGIs regions was significantly different from each other, PMR of the ASD sample, who had a loss of 1044.756 Kb was 40.6% and 82.3% in CpGI-1 and CpGI-2, respectively. This is generally considered a high methylation ratio compared to others. These findings are consistent with those of previous study which revealed that DNA methylation increases across CpGIs in copy number deletion region[10]. In contrary to Sun et al[10] we found out that the ASD sample that had a loss of 571.927 Kb showed a reduction in PMRs in two CpGIs compared to others, which were 0% in the first CpGI and 36.6% in the second CpGI. In addition, the study by Robinson et al[29] indicated a deletion of about 70 Mb on chromosome 13 but no subsequent change in methylation[65].

Conclusions

This study was divided into two sets of experiments: aCGH and Methylight qPCR in order to investigate the correlation between CNV and DNA methylation in chromosome 14 of ASD children. However, the aCGH result indicated CNVs on six cytobands in chromosome 14 for 13 out of 14 autistic samples and 4 normal samples with different sizes. Furthermore, these CNVs affected many significant genes that play important role in biological processes such as apoptosis, embryonic development, proliferation, migration, and signaling. The most striking result from the data is that the OR genes are strongly correlated to ASD cases. Furthermore, aberrant methylation of two CpGIs that correlated with CNV in chromosome 14 has been detected among ASD. The explanation for the cause of the difference in methylation levels between autistic and normal samples remains unclear, and further research with advanced techniques and more samples is needed.

However, more research with increased sample size is needed to validate the results of CNVs in chromosome 14 and their correlations to methylation levels. In terms of directions for future study, further advanced research investigating the association between OR genes and ASD is required.

Conflict of Interest

The authors have no conflicts of interest to declare.

Authors' Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Institutional Review Board Statement

This study was designed with correspondence to the codes of the guidelines for Ethics Committee of Biomedical Research-Centre of Excellence in Genomic Medicine Research at King Abdulaziz University, Ethical Approval Number (02-CEGMR-Bioeth-2018). The study was executed in consensus with the guidelines followed in King Fahd Center for Medical Research, KAU, Jeddah, Saudi Arabia, which were in accordance with declaration of Helsinki.

Informed Consent Statement

Informed consent forms were signed by the parents of the participants.

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