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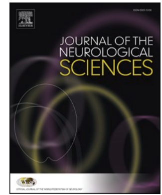

Iron metabolism in autism spectrum disorder; inference through single nucleotide polymorphisms in key iron metabolism genes

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ABSTRACT

Autism spectrum disorder (ASD) is a heterogeneous group of neurodevelopmental problems with various genetic and environmental components. The ASD diagnosis is based on symptom expression without reliance on any biomarkers. The genetic contributions in ASD remain elusive. Various studies have linked ASD with iron. Since iron plays a crucial role in brain development, neurotransmitter synthesis, neuronal myelination and mitochondrial function, we hypothesized that iron dysregulation in the brain could play a role and contribute to the pathogenesis of ASD. In this study, we investigated single nucleotide polymorphisms in ASD in various iron metabolism genes, including the Transferrin Receptor (*TFRC*) gene (rs11915082), the Solute Carrier Family 11 Member 2 (*SLC11A2*) gene (rs1048230 and rs224589), the Solute Carrier Family 40 Member 1 (*SLC40A1*) gene (rs1439816), and hepcidin antimicrobial peptide (*HAMP*) gene (rs10421768). We recruited 48 patients with ASD and 88 matched non-ASD controls. Our results revealed a significant difference between ASD and controls in the G allele of the *TFRC* gene rs11915082, and in the C allele of the *SLC40A1* gene rs1439816. In silico analysis demonstrated potential positive role of the indicated genetic variations in ASD development and pathogenesis. These results suggest that specific genetic variations in iron metabolism genes may represent part of early genetic markers for early diagnosis of ASD. A significant effect of SNPs, groups (ASD/control) as well as interaction between SNPs and groups was revealed. Follow-up post hoc tests showed a significant difference between the ASD and control groups in rs11915082 (*TFRC* gene) and rs1439816 (*SLC40A1* gene). Backward conditional logistic regression using both the genotype and allele data showed similar ability in detecting ASD using allel model (Nagelkerke $R^2 = 0.350$ $p = 0.967$; Variables: rs1439816, rs11915082) compared to genotype model (Nagelkerke $R^2 = 0.347$, $p = 0.430$; Variables: rs1439816 G, rs1439816 C, rs10421768 A). ROC curve showed 54% sensitivity in detecting ASD compared to 47% for the genotype model. Both models differentiated controls with high accuracy; the allele model had a specificity of 91% compared to 92% for the genotype model.

In conclusion, our findings suggest that specific genetic variations in iron metabolism may represent early biomarkers for a diagnosis of ASD. Further research is needed to correlate these markers with specific blood iron indicators and their contribution to brain development and behavior.

1. Introduction

Autism spectrum disorder (ASD) includes a set of early onset neurodevelopmental aberrations with an estimated worldwide prevalence of 1 per 100 children [33]. ASD patients express a wide range of symptoms and difficulties primarily in social interaction, restricted,

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repetitive interests and behaviors [1]. The early symptoms of ASD are usually identified by the age of 1 to 3 years old [25]. Accumulating evidence has shown a significant role of environmental factors in the pathogenesis of ASD [14,21]. Additional factors associated with ASD include advanced paternal age [19] and exposure to some environmental mutagens during pregnancy including mercury, cadmium and nickel [18]. In addition to environmental factors, ASD is confirmed to be influenced by multigenic factors that are highly heterogeneous, with the average case being a product of many susceptibility-increasing genetic variations [8,32]. Furthermore, patients with ASD exihibit various changes in multiple peripheral markers, such as iron deficiency [5,12,15,20].

Iron is a vital metal in all living organisms and contributes to many vital processes [24]. In the brain, iron is essential for brain development, neurotransmitter synthesis, neuronal myelination and mitochondrial function. Iron metabolism is controlled by many key proteins, including transferrin receptor 1(*TFRC*), solute carrier family 11 member 2 (*SLC11A2*), solute carrier family 40 member 1(*SLC40A1*), and hepcidin antimicrobial peptide gene (*HAMP*), as illustrated in Fig. 1. Due to the significant and crucial role iron plays in brain development, we hypothesized that brain iron dysregulation may play a role in ASD pathogenesis. In particular, we focused on five single nucleotide polymorphisms that have been significantly associated with known neurological disorders including Alzheimer's and Parkinson's disease. The rs11915082 polymorphism in the *TFRC* gene [17] is suspected to affect regulation and level of expression of the transferrin receptor 1 gene [30]. Further, rs1439816of the *SLC40A1* gene that encodes ferroportin1 has been linked with Alzheimer's disease risk [11]. Both rs1048230 and rs224589 in the divalent metal transporter 1 have been shown to be associated with Parkinson disorder [26,30]. Finally, rs10421768 is located in the promoter region of the *HAMP* gene that has been linked with ASD [28] (CITE) as well as with iron overload in thalassemia major patients. In this study, we investigated the association between these single nucleotide polymorphisms (SNPs) and ASD.

2. Methods

2.1. Subjects and samples

We recruited 48 male Palestinian patients with ASD with a median age of 8 years old (4–14 years old). The control group constituted of 88 healthy young Palestinian males with no signs of any neurological or other disorders and symptoms were included as controls. Since patients came from different localities in the country, control particiants were recruited from the same localities. Clinical diagnosis of ASD was performed for most patients (40) based on DSM-5 criteria by specialists in the field (neurologists and psychiatrists) in private clinics and specialized societies. A portion of the patients (8) -those born before 2013 - were initially diagnosed based on DSM-IV criteria, and diagnosis were later confirmed by DSM-5 criteria. The behavioral differences among all patiens were recorded. Both patients and control subjects were recruited from the same geographical area covering various districts in the West Bank, Palestine. Guardians of all patients were fully informed of the objectives and course of the research project and gave written informed consent for their children to participate in the study. All relevent clinical and neurological complications of patients subjects were obtained from their medical records and gurdians description but couls not be included in the molecular association evaluation due to limited number of patiens who share similar signes and symptoms.

2.2. Genotyping

Whole blood samples (3–5 ml) were collected from all participating subjects in EDTA tubes and genomic DNA was extracted using commercial kit (Epicenter, USA) according to manufacturer guidelines. Purified DNA was stored at -30 °C and the selected SNPs in the indicated genes awere based on their reported link with neurological disorders as meniond before. Genotyping was determined by Sanger's sequencing for SNPs rs11915082, rs1048230, rs224589, and rs1439816 variants while PCR-RFLP was used for rs10421768 variants determination. PCR reactions were run in 25ul volume containing 12.5 μl of 2× ready mix (Promega, USA), 1 μl template genomic DNA (about 200 ng/μL), and 1 μl of forward and reverse primers (final concentration of 10 μM). The PCR reactions mixes and thermal cycles protocols including the relevant primers sequences are described in Table 1 and the reactions were run on a FlexCycler2 thermocycler (Analytik Jena, Germany). In the PCR-RFLP analysis, the amplicons were digested using HpyCH41V allele-specific restriction enzyme. Each reaction contained 10 μl PCR product, 1.5 μl 10 x enzyme buffer, 0.2 μl (2 units of enzyme), and 3.3 μl distilled water. The reaction mixture was incubated for 24 h at 37 °C and the DNA digestion pattern was analyzed on 2% agarose gels. Sanger's sequencing was performed using BigDye™ Direct Cycle Sequencing Kit (Thermo Fisher scientific, USA) and analyzed on Applied Biosystems 3500 Genetic Analyzer.

2.3. Statistical and in silico analysis

Allele and genotype frequencies were calculated using SPSS software and SHEsis online tool [16,27]. Overall differences in allele and genotype frequencies between ASD patients and non-ASD individuals were

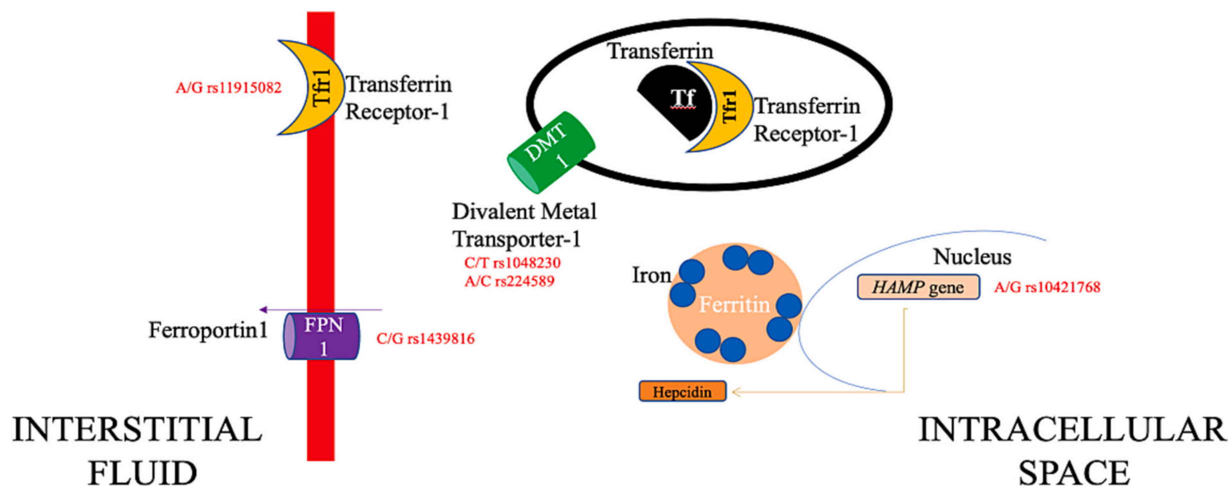

Fig. 1. Polymorphisms in various elements of iron membrane/intracellular control genes.

Table 1
PCR protocols and primers' sequences.

SNP	Initial denaturation5 min)(	Cycling			Final extension (5 min)	Forward primer	Reverse primer	Product size
		Denaturation30 s	Annealing30 s	Elongation45 s				
Rs11915082	94 °C	94 °C	56 °C	74 °C	74 °C	GTCACTTCCTGAGGCACGTA	CGCAGTGCAATATCCAACAT	306
Rs1048230	94 °C	94 °C	56 °C	74 °C	74 °C	TCCCATTCTTCTGAGGTCTCTC	AGACCACAACCATGCCTCTG	366
Rs224589	94 °C	94 °C	56 °C	74 °C	74 °C	TGTGAGGCTGGATTTTGTTG	AGATTTTGCACATGACCTGCT	483
Rs1439816	94 °C	94 °C	58 °C	74 °C	74 °C	TGGGGAAAGATCTTCGATG	GTACGTGGTTTGTCCTGCAA	367
Rs10421768	94 °C	94 °C	58 °C	74 °C	74 °C	CCCAGGCTAGTCTTGAACTTCTG	AGGGAACACTAGATAGCCCTGAG	244

evaluated by Chi-Square (χ^2) test for independence or Fisher's exact test. Genotypes were evaluated using a -model ANOVA with the different SNPs as the within subjects variables, and the as the between 0 subjst variable. A p value less than an $\alpha = 0.05$ was considered statistically significant. Bonferroni correction of the α level was applied when relevant. The functional consequences of SNPs that showed significant association with ASD were investigated by in silico tools including RegulomeDB and HaploReg V4.1 [7,29]. RegulomeDB scores SNPs functionally based on their existence in a DNAase hypersensitive site or transcription factor binding sites[7]. Scores range between 1 and 6, in which score 1 shows that a SNP has strong evidence of being regulatory, and 6 means it has least evidence of being functional [7]. HaploReg V4.1 was used to investigate whether the SNPs may have regulatory functions.

3. Results

3.1. Association of SNP allele and genotype frequencies with ASD

Table 2 shows significant differences in allele frequencies between the ASD and control groups for SNPs rs11915082 (*TFRC* gene) ($p = 0.008$) and rs1439816 (*SLC40A1* gene) ($p \leq 0.0001$). The G allele of rs11915082 was associated with increased ASD risk (OR = 2.06, 95% CI = 1.19–3.55, $p = 0.008$). Similarly, the C allele of rs1439816 was associated with increased disease risk (OR = 5.13, 95% CI = 3.00–8.79, $p \leq 0.0001$). Both rs224589 and rs1048230 (*SLC11A2*) and rs10421768 showed no significant differences in allele frequencies between cases and controls. All tests had a Bonferroni corrected $\alpha = 0.01$.

For genotypes frequencies (Bonferroni corrected $\alpha = 0.01$), the results indicated asignificant difference between the ASD and control groups rs1439816 ($p \leq 0.002$) as shown in Table 3. The CC genotype variant of rs1439816 was more frequent among subjects with ASD compared to controls (47.9% vs. 8%, $p \leq 0.000$), which is in agreement with the C allele frequency that showed significant association with increased ASD risk. Conversely, rs10421768 showed no significant difference at the level of allele frequency between subjects with ASD and controls. A mixed-model ANOVA with the five SNPs as within-subject variables and the group (ASD/control) revealed a significant effect of SNPs ($p < 0.001$), a significant effect of group ($p < 0.001$), and a significant interaction between SNPs and group ($p < 0.001$). Follow-up post hoc independent-samples *t*-tests to explore the significant interaction showed a significant difference between the ASD and control groups

Table 2
Statistical analysis of allele and genotype frequencies.

SNP	Allele	Patient (%) ($n = 48$)	Control (%) ($n = 88$)	P Value	OR(95% CI)
rs11915082	G	71(74.0%)	102(58.0%)	**0.008**	2.06 (1.19–3.55)
	A	25(26.0%)	74(42.0%)		0.48 (0.28–0.83)
rs1048230	T	74(77.1%)	140(79.5%)	0.635	0.86 (0.47–1.57)
	C	22(22.9%)	36(20.5%)		1.15 (0.63–2.10)
rs224589	A	32(33.3%)	53(30.1%)	0.584	1.16 (0.68–1.97)
	C	64(66.7%)	123(69.9%)		0.86 (0.50–1.46)
rs1439816	C	65(67.7%)	51(29%)	**>0.001**	5.13 (3.00–8.79)
	G	31(32.3%)	125(71.0%)		0.19 (0.11–0.33)
rs10421768	A	75(78.1%)	145(82.4%)	0.393	0.76 (0.41–1.41)
	G	21(21.9%)	31(17.6%)		1.30 (0.70–2.43)

Values in bold indicate significant difference.

Table 3
Statistical analysis of genotype frequencies.

SNP	Genotype	Patient (%) (n = 48)	Control (%) (n = 88)	P Value
rs11915082	GG	27(56.2%)	31(35.2%)	**0.041**
	AG	17(35.4%)	40(45.5%)	
	AA	4(8.3%)	17(19.3%)	
	TT	29(60.4%)	53(60.2%)	
rs1048230	TC	16(33.3%)	34(38.6%)	0.223
	CC	3(6.2%)	1(1.1%)	
	AA	6(12.5%)	4 (4.5%)	
rs224589	AC	20(41.7%)	45(51.1%)	0.194
	CC	22(45.8%)	39(44.3%)	
	CC	23(47.9%)	7(8%)	
rs1439816	CG	19(39.6%)	37(42%)	**<0.001**
	GG	6(12.5%)	44(50.0%)	
	AA	32(66.7%)	58(65.9%)	
rs10421768	AG	11(22.9%)	29(33.0%)	**0.028**
	GG	5(10.4%)	1(1.1%)	

Values in bold indicate significant difference.

in rs11915082 (*TFRC* gene) and rs1439816 (*SLC40A1* gene).

We applied conditional backward logistic regression using both the genotype and allele data to examine the significance of our data in differentiating ASD from controls. The allele model showed similar ability in detecting ASD (Nagelkerke $R^2 = 0.350 p = 0.967$; Variables: rs1439816, rs11915082) compared to the genotype model (Nagelkerke $R^2 = 0.347$, $p = 0.430$; Variables: rs1439816 G, rs1439816 C, rs10421768 A). ROC curves in Fig. 2 illustrate the difference between the allele and genotype models in detecting ASD and controls. The allele model showed 54% sensitivity compared to 47% for the genotype model. Both models differentiated controls with high accuracy, with the allele model showing a specificity of 91% compared to 92% for the genotype model.

3.2. *In Silico analysis of the significant variants in the indicated genes*

In silico analysis using RegulomeDB and HaploReg V4.1 was carried out for SNPs that showed significant statistical association with ASD. For rs11915082, RegulomeDB analysis showed a score of 4, indicating a lack of evidence that this SNP has a regulatory consequence. Alternatively, HaploReg analysis showed that rs11915082 affects the binding sites of regulatory factors CHD2, SRF, and ZBTB33. Regarding rs1439816, RegulomeDB analysis showed that rs1439816 SNP has also a score of 4. However, HaploReg analysis showed that this variation disrupts two regulatory motifs including the binding sites of Eomes and SREBP. Finally, in silico analysis using RegulomeDB showed that rs10421768 variant has a score of 2b, indicating that this variation is likely to affect a transcription factor binding. In addition, the HaploReg analysis showed that this SNP affects the binding sites of a group of factors including Myc, E2A, and HIF1.

4. Discussion

Iron is found in high concentrations within the brain where it plays essential roles in different cellular processes including neurotransmitter synthesis, myelination of neurons and mitochondrial function. Brain iron is highly regulated by different mechanisms mediated by various key gene products [24]. Oxidative damage is considered as one of the contributing factors in ASD pathogenesis [22]. Brain oxidative damage could be enhanced by excess free iron within the brain. Increased levels of non-transferrin bound iron may accumulate due to deregulation in the functional expression of iron homeostasis proteins including transferrin (Tf), transferrin receptor (TfR1), hepcidin antimicrobial peptide gene (HAMP), Ferroportin (FPN) and divalentmetal-ion transporter1 (DMT1). Decreased levels of transferrin and ceruloplasmin have been documented in blood samples from autistic patients [9,23,31,34]. The possibility that ASD risk can be altered due to individual genetic variations that affect and modify their susceptibility cannot be excluded [2]. Even in the absence of toxic iron levels, iron could contribute to autism etiology due to genetic susceptibility. In the present study, the association of specific single nucleotide polymorphisms in specific genes directly involved in iron metabolism including rs11915082 in*TFRC* gene, rs224589 in *SLC11A2* gene (DMT1), rs1048230 in *SLC11A2* gene (DMT1), rs1439816 in *SLC40A1* (FPN) and rs10421768 in *HAMP* gene (Hepcidin) and ASD risk was investigated. The results evidently showed rs11915082, rs10421768, and rs1439816 to be risk genetic factors associated with ASD risk. The transferrin receptor (TfR1) together with transferrin (Tf) mediates the delivery of Tf-bound iron into cells. The rs1191508 SNP has been reported to be associated with age-related

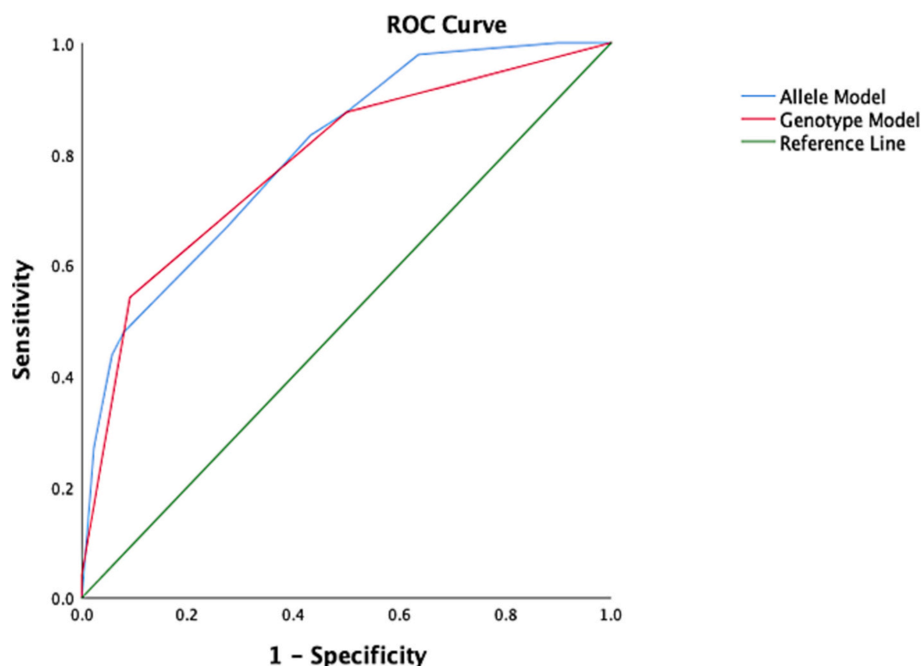

Fig. 2. ROC curves of conditional backward logistic regression analysis of allele vs. gnotype detection of ASD.

macular degeneration [30]. The present data demonstrate that the rs11915082 genetic variant in the TFRC gene is associated with ASD pathogenesis, with the GG genotype being more frequent in the patient group compared to the control group. Defifnitely, this will require direct investigation on the relationship between iron metabolism-related gene polymorphisms and various specific hematological parameters including serum iron, ferritin levels, transferring levels and total iron blood capacity (NTBC). The results indicate a significant role of this SNP in ASD pathogenesis through disruption of iron metabolism in the brain. Ferroportin (FPN) mediates the export of iron from cells encoded by the *SLC40A1* gene. A study on rs1439816 variant reported the C allele as a risk factor of Alzheimer's diseas. This allele was significantly associated with decreased expression of the *SLC40A1* gene in those patients resulting in increased cellular Fe levels [11]. Our results similarly showed the C allele of this variant was significantly associated with ASD patients compared to controls. Our study showed significant difference in ferroportin genetic variant rs1439816 frequencies between controls and patients subjects which indicates a possible role of this SNP in ASD pathogenesis. This will require further investigation to correlate the indicated genotypre variation with specifc Iron metabolism indicateors as mentioned above which will the development of a model on the contribution of iron fluctuation to brain tissue functions in autistic patients compared to healthy subjects.

Hepcidin is encoded by the hepatic antimicrobial protein gene *HAMP* and is considered a central regulator of systemic iron homeostasis through the control of FPN1 expressions [13]. The rs10421768 polymorphism (c.-582 A < G) is located in the promoter region of the *HAMP* gene. This variation has been reported to be associated with many disorders which affect a conserved non-coding transcriptional box of the *HAMP* gene promoter and it may affect binding affinity to transcriptional factors or the response to these factors. In addition, it is suggested that variation in the activation of the *HAMP* gene expression by both upstream regulatory factor 1 and 2 (USF1/USF2) and cMyc/Max heterodimers that occur through E box within the promoter, can alter the regulation of *HAMP* gene and hepcidin function in iron homeostasis [3,4]. This polymorphism may constitute a risk factor for ASD through increased iron levels in patients with ASD. Our data indicated a significant role of *HAMP* gene rs10421768 variant in the pathogenesis of ASD with significant differences in genotype frequencies between patients and control groups, however, no significant difference was detected in allele frequencies between the two groups that can be explained by the relative small size of the patients group.

The divalent metal transporter 1 (DMT1) is encoded by the solute carrier family 11 member2 gene *SLC11A2* and is ubiquitously expressed in different tissues including brain and liver [35]. DMT1 is involved in iron absorption and transport. The rs1048230 variant is synonymous and located in the coding region of the *SLC11A2* gene [17] but does not result in amino acid substitution. This SNP was reported as a benign variant when correlated with iron overload in hypochromic microcytic Anemia (Accession: VCV000309312.2) [36]. The TT genotype and T allele were documented to be associated with Parkinson disorder [26]. Regarding the rs224589 polymorphism (IVS4 + 44C < A), which is an intronic variant located in intron 4, the C allele was reported to be significantly associated with Wilson's disease, age related macular degeneration and Parkinson disorder [37–39]. It is speculated that the rs224589 variant may affect alternative splicing or constitutive splicing of the *SLC11A2* gene through the corruption of splicing regulatory cis-elements, which can result in incorrect isoforms of DMT1 [38,40,41]. Furthermore, since this SNP is located in an intronic region, it might affect transcription, post-transcription, and ultimately mRNA translation of the *SLC11A2* gene. Our results showed that both the rs1048230 and the rs224589 variants located in *SLC11A2* gene were not correlated with ASD pathogenesis as both alleles and genotypes frequencies were not significantly different between patient and control groups.

This study represents the first study to draw direct attention towards the role of iron dysregulation in ASD pathogenesis. Effects of genetic variations in iron homeostasis genes and iron homeostasis is evident [6]. Our study focused on molecular genetic analysis; however, it is highly recommended to expand this investigation on the relationship between iron metabolism-related genes polymorphisms and various specific hematological parameters including serum iron, ferritin levels, transferrin levels and total iron blood capacity (TIBC). Our results strongly indicate the need to extend this work to directly evaluate the levels of blood iron status including transferrin, ferritin and NTBI levels in normal subjects and individuals with ASD to develop a model on the contribution of iron fluctuation to brain tissues functions in autistic patients compared to healthy subjects. In addition, the application of individual SNPs association studies is considered limited due to the low penetrance of individual variants and the difficulty in their expressivity. Therefore, individual haplotypes association is considered more valuable and relevant following direct link of these genetic variations with a specific phenotype [10]. The in silico analysis results indicated the SNPs which showed significant correlation with ASD are likely to influence binding of several major transcription factors. This provides an important tool for unraveling the possible mechanism for the functional effect of these variants on brain Fe status. This can be examined through identification of relevant binding sites for these factors in the SNPs vicinity and investigation of their functional significance.

Our findings should be considered in the context of a number of limitations. First, the number of subjects with ASD in our study was relatively small. This was influenced by challenges in recruiting patients accurately diagnosed with ASD based on standardized criteria, and by the limited number of specialists and professional societies in our fairly small population. In addition, recruitment of subjects was also tempered by social concerns of the involved families to participate in these studies. While we acknowledge the need to expand this study to include more patients, especially with extended investigation of relevant blood iron parameters in ASD, the fairly strong association between the three indicated SNPs with ASD within the current number of patients provides preliminary confidence of a link between these variables. Second, despite our best efforts to match the healthy control patients to the patients with ASD, there was a significant difference in age range between the two groups (4–14 years and 18–20 years, respectively). While we don't perceive any immediate compelling reasons this would impact our results, it is possible that factors unknown to us at the time of this study could have impacted the results based on the different age ranges of the cases versus controls. However, the fairly strong significant differences of the association between the 3 indicated SNPs provides support for the significant link of these molecular markers with the development of the disease. These results provide the basis for expanded investigation of specific blood iron markers including free serm iron, serum iron binding proteins, the function of specific vital Fe- containg enzymes and the expression status of the indicated genes in these patients compared to control subjects and their potential role in the development of the disease.

Iron is critical for proper neurodevelopment and its metabolism in the brain is tightly regulated. Therefore, structural and functional modulation of iron metabolism genes may represent one fundamental factor linked to ASD development. The results generated from this study give significant insights into the association of specific genetic variants in major iron metabolism genes and ASD development, providing further support to the role of iron in neurodevelopment and the implication of its dysregulation on ASD development and pathogenesis. This study provides evidence for new novel genetic markers of brain iron metabolism for ASD susceptibility that have the potential to serve as additional major genetic markers for early diagnosis of ASD and early clinical intervention.

Author contributions

SR genotyped the samples and edited the manuscript; SN contributed to the design of genotyping protocols and processing of samples and

edited the manuscript; SN and MMH designed the research framework and identified target genes; KB conducted statistical analysis and edited the manuscript; MMH conducted statistical and machine learning analysis and edited the manuscript, HMD organized the collection of patients and control sampls, wrote the first draft of the manuscript, designed of genotyping protocols, and collected the data. HMD and MMH co-supervised the inception and execution of the project.

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