
Gebril OH*1 and Meguid NA2

*1,2Department of Children with Special Needs, Medical Division, National Research Centre, Cairo, Egypt.

Autism is showing increased incidence worldwide, with many theories being studied as risk factors, with oxidative stress dysregulation being widely studied. Cellular Iron metabolism is considered a pivotal factor in regulating oxidative stresses and hence neurotoxicity. In this study, ferroportin gene (SLC40A1) Q248H polymorphism, which is a functional polymorphism was studied in autism children and control in a preliminary study. Our results do not show increased incidence of this polymorphism in autism along with absence of changes in blood ferritin levels. Significant decreased serum iron levels in ASD children compared to control children was noticed and this is explained by nutritional factors in these patients. To conclude, our study does not support a role of cellular iron export disturbances in increasing oxidative stress in autism patients, and hence other mechanisms would be responsible. Other extended proteomic analysis is warranted in neurodevelopmental disorders and autism to elucidate the pathophysiological mechanism.

Keywords: Iron, oxidative stress, cellular export, ferritin, haemochromatosis

INTRODUCTION

Many studies showed increased prevalence of iron deficiency anaemia and changes in iron parameters in autism spectrum disorder (ASD) (Herguner et al., 2012). Clinical survey of ASD children under the age of 6 years and their age-matched controls revealed evidence of abnormal markers of thiol metabolism, as well as a significant alteration in deposition of several heavy metal species, particularly iron, arsenic, mercury, and copper in hair samples. While exact iron metabolism and regulating proteins in the brain are still under debate, a disturbance of cellular iron exportation and hence increased oxidative stresses have been linked to many neurodegenerative disorders either as etiological or potentiating factor (Gebril et al., 2011a; Obrenovich ME et al., 2011).

Increased oxidative markers in autism had been evidenced and thus a link to iron metabolism disturbance and risk for autism had been suspected (Rossignol et al., 2014; Rose et al., 2014). Interestingly, a recent proteomic study in blood of autism patients have shown increased expression of proteins responsible for oxidative stress namely apolipoproteins (Apos), and serum paroxanase/arylesterase 1 (PON1) (Ngounou Wetie et al., 2014). In addition a recent review have elucidated many pathological features of neurodegeneration in autism as neuronal cell loss and activated brain microglia and astrocytes (Kern et al., 2013). One of the target brain proteins which is thought to play a role in regulating cell iron haemostasis and oxidative stresses is ferroportin (SLC40A1) (Skjørringe et al., 2012). Ferroportin protein is thought to be a major iron exporter protein via abluminal border in choroid plexus and thus is responsible for iron efflux (Rouault et al., 2009), Its function is through interaction with another brain iron regulating protein; haemochromatosis (HFE). We have studied previously HFE gene polymorphisms in autism and no significant association was found (Gebril and Meguid, 2011).

*Corresponding author: Ola H. Gebril, Department of Children with Special Needs, Medical Division, National Research Centre, 12622 Elbehoos street, Dokki, Cairo, Egypt, E-mail: olahossny@hotmail.com, meguidna@yahoo.com, Tel.: +2 01157583452

Ferroportin SLC40A1 gene and autism
The cDNA 744G toT substitution in exon 6 of the ferroportin gene, results in the replacement of glutamine with histidine at position 248 (Q248H). It has been studied in many African populations, mainly in association with a tendency to iron loading (Gordeuk et al., 2003; McNamara et al., 2005)). Cellular studies indicate that, unlike the ferroportin mutations that are associated with macrophage iron overload in white families— e.g. A77D and V162del (Wallace et al., 2002)—the Q248H mutation has not been reported to decrease the macrophage membrane expression of ferroportin or to influence the cellular expression of transferrin receptors or the cellular ferritin content in the absence of inflammation (Schimanski et al., 2005). This is a pilot study to look for frequency of SLC40A1 gene Q248H mutation in autism children and control. This would represent a preliminary guide for the role of SLC40A1 gene in increasing the risk for ASD which shows increasing worldwide prevalence.

MATERIALS AND METHODS

Twenty five autism cases were selected with age range 5–15 years (6.6 years ± 4.4). A comparable control group included twenty four normal, age matched children. Any children suffering from chronic disorders or perinatal insults, for example, perinatal hypoxic ischaemic insults – were excluded from the study. Each family of a child with ASD was asked to call another family from the same area with a normal child. All autism patients are males and hence the gender of control children are male. All the families were recruited from the Autism Disorders Clinic, Medical Research Center of Excellence, National Research Center. Exclusion criteria included the following: Children with visual, hearing, motor impairment, identified metabolic, genetic and neurological disorders. Autistic children were diagnosed using Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV-R (American Psychiatric Association, 2000), and Childhood Autism Rating Scale (CARS) (Schopler et al., 1980).

Caregivers consent was obtained from all studied cases and control and the study procedure was explained. Institutional ethical approval was obtained for the study.

Detection of SLC40A1 gene polymorphism

Venous Blood (2ml) was collected in EDTA containing tubes for DNA extraction. Genomic DNA was isolated by standard procedures using the DNA extraction kit (DNeasy Blood & Tissue Kit) (Qiagen). Exon 6 of ferroportin was amplified by using a set of primers encompassing portions of the introns that flank exon 6 according to a previously mentioned method (Kasvosve et al., 2005). The 392-base pair (bp) product was digested with PvuII enzyme (MBI Fermentas, Hanover, MD), and the resulting DNA fragments (252 and 140 bp) were fractionated on 3% agarose gel and detected with ethidium bromide. Ferritin level and serum iron was determed in blood using colorimetric procedure. All Statistical analysis was done using GraphPad Prism 5.0. Chi-square ($\chi^2$) testing was used to evaluate the association between ferroportin gene polymorphisms and autism. P-values of 0.05 were considered statistically significant.

RESULTS

The present study included 11 patients with severe autism (CARS 39-60) and 14 patients with mild to moderate autism (CARS 30-38). Serum iron in autism children was 65 ± 18.5 µg/dL and in control it was 75 ± 15.3 µg/dL ($p=0.04$). The blood ferritin level in autism and control children ranged from 25–49 ng/ml. No significant difference between both groups was detected, with a p value of 0.4. No effect of Q248H genotype on ferritin level was detected ($P=0.6$).

The Q248H PCR products (210 bp) were used for genotyping (Fig. 1). In the control group, one heterozygous (Q248H) is detected and the rest are wild heterozygous.
type Q248H hence the H allele frequency is 1/48 (2%) and the Q allele frequency is 98%, while the mutation is not found in autism patients, frequency of Q allele is 100%. Allele frequencies are in Hardy Weinberg equilibrium.

**DISCUSSION**

Our results do not show a link between Q248H polymorphism of SLC40A1 gene and risk for autism, these findings do not support increased brain intracellular iron level as a cofactor for potentiating or enhancing oxidative stress in autism. These data goes in agreement with our previous study that included HFE gene polymorphisms in autism (Gerbil et al., 2011) with comparable sample number. These two studies include genes responsible mainly for controlling iron level in brain and central nervous system. Several studies found evidences for oxidative stress in autism patients; 4-hydroxynonenal protein adducts, as a marker of lipid peroxidation-induced protein damage was increased in blood. Intra-erythrocyte and plasma non protein bound iron levels were significantly increased (1.98- and 3.56-folds) in autistic patients' blood, as compared to controls (Pecorelli et al., 2013). Also intra-erythrocyte non-protein-bound iron, F2-isoprostanes was evident in classic Rett syndrome (De Felice et al., 2009), which shares many symptomatic and pathophysiological features with autism. This points to the oxidative stress dysregulation in plasma and red blood cells in autism, while no previous evidence of disturbed iron in central nervous system was found.

Serum iron was decreased in autism patients compared to control with a p value= 0.04 with no difference in ferritin levels between both groups. Previous studies showed decreased blood iron parameters and ferritin in ASD patients (Youssef et al., 2013), this could be attributed to nutritional deficiencies in these children being found in other children with neurodevelopmental disorders (Sidrak et al., 2014). Another study included more than two thousand patients with iron deficiency anaemia with various psychiatric disorders showed increased prevalence in ASD, unipolar depressive disorder, anxiety disorder, attention deficit hyperactivity disorder, developmental delay and mental retardation (Chen et al., 2013). We suspect that increased oxidative stress in autism is due to impairment in function of enzymes responsible for getting rid of oxidative products, as this is supported by a recent proteomic study that showed increased levels of apolipoproteins and PON1, involved in preventing oxidative damage (Ngounou Wetie et al., 2014). This is supported by total antioxidant status, non-enzymatic (glutathione and homocysteine) and enzymatic (catalase, superoxide dismutase, and glutathione peroxidase) antioxidants, and lipid peroxidation in plasma and erythrocytes in Asperger syndrome children (Parellada et al., 2012). The importance of our study is that it is the first study to include SLC40A1 gene polymorphism in neurodevelopmental disorders, further proteomic studies for iron regulating proteins is warranted. The frequency of SLC40A1 gene polymorphism in our control samples is less than previously shown in other populations (0.02). The aggregate Q248H frequencies in African-Americans and native Africans differed significantly (0.0525 vs. 0.0946, respectively (Barton et al., 2007) and the frequency of the Q248H polymorphism was greater in African American men in another study (Rivers et al., 2007), this points to various polymorphism prevalence in different populations. This study is the first study to our knowledge to explore Q248H polymorphism in Egyptians and similar ancestors in Middle East region. Although, the number of ASD cases can be extended in further studies in similar populations with gene expression analysis, our results highlight the rarity of this functional polymorphism in our population. We could not study the effect of gender on Q248H, as all included autism cases are males and so matched male control were included.

To conclude, although oxidative stress has been implicated for a role in ASD, the role of iron is not clear and we suspect other factors and enzymes as the biological background. Other extended studies that include iron proteins expression in autism children are needed to confirm these data.

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