Elevated Plasma X-Linked Neuroligin 4 Expression is Associated with Autism Spectrum Disorder

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Short title: Plasma Neuroligin 4 and Autism Spectrum Disorder

Keywords: Autism spectrum disorders · Neuroligin 4 · Childhood autism rating scale · Social responsiveness scale · Synaptic function
Highlights of the Study:

- This is the first study of a possible correlation between plasma neuroligin 4 and the degree severity of autism.
- The increased plasma levels of neuroligin-4 suggest that it may play a role in the pathogenesis of autism.
- Neuroligin-4 could possibly be a valuable biomarker for autism.
Abstract:

Objectives: In this study, we compared plasma levels of NLGN4 in children with autism versus matched healthy controls to examine a possible correlation between plasma level of NLGN4 and the degree of severity of autism, and social impairment in the autistic patients. Subjects and Methods: 88 autistic patients aged 3 – 12 years and 33 age-and sex-matched controls aged 3 - 9 years were recruited. Plasma levels of NLGN4 were determined using a commercial enzyme-linked immunoassay (ELISA). The Childhood Autism Rating Scale (CARS) and the Social Responsiveness Scale (SRS) were used to assess cognitive dysfunction and social impairment in autistic patients. Results: plasma levels of NLGN4 were significantly higher ($p = 0.001$) in autistic children in comparison to healthy controls. In spite of the alteration in the level of NLGN4 among the subgroups of autistic children, no correlation between plasma concentration of NLGN4 and cognitive problem, or social impairment was observed ($p > 0.05$). Conclusion: Increased plasma concentrations of NLGN4 may play a role in the pathogenesis of autism, and it could be a valuable biomarker for autism. Further studies with larger sample sizes are warranted to validate this finding, and also to explore the potential links between NLGN4 and features of autism.
Introduction

Autism is a common neurodevelopmental disorder characterized by impaired social interaction, communication problems and stereotyped behaviors, together with sensory abnormalities [1 - 2]. The etiology of this disorder is still unresolved, with genetic and environmental factors implicated in the pathophysiology of autism [1, 3, 4]. The current estimated prevalence of autism is approximately 1%, and the male-to-female is 4:1 [5].

Neuroligins (NLGNs) are adhesion molecules that are expressed in postsynaptic neurons. They interact with neurexins (NRXN) expressed in presynaptic neurons [6, 7]. Five genes encoding neuroligin proteins have been identified in the human genome (NLGN1, NLGN2, NLGN3, NLGN4X, NLGN4Y), while only NLGN1, NLGN2, NLGN3, and NLGN4 genes have been identified in other mammalian species like mice [8]. NLGNs are composed of an extended N terminal extracellular domain containing a large esterase homology domain necessary for the activity of neuroligins, a short O-glycosylated region linked to a single transmembrane domain, and a short C-terminal intracellular domain [9]. Several studies have suggested that levels of NLGNs are regulated by proteolytic processing; NLGNs are proteolytically cleaved in a sequential manner, as a result of basal and also activity-dependent excitatory transmission [10, 11]. NLGNs are initially cleaved by metalloproteases, which are responsible for the shedding of the ectodomain at the juxtamembrane stalk region, releasing the soluble form of NLGNs (sNLGNs) and a membrane-tethered C-terminal fragment (CTF). CTF then undergoes a subsequent cleavage mediated by γ-secretase to release the intracellular domain (ICD). As the proteolytic processing takes place on the cell surface, it leads to the removal of NLGNs from the cell surface and decreases their activity [10, 11].
The neuroligin–neurexin interaction is hypothesized to play a role in synaptic function, formation and stabilization. NLGN1, NLGN3, and NLGN4 are localized at excitatory glutamatergic axons, while NLGN2 is localized at inhibitory gamma-aminobutyric acid (GABA) axons [6, 12].

Mutations of several synaptic have been implicated in the molecular pathogenesis of neurodevelopmental disorders, including autism. These mutations may affect the synaptic pathway structure and function [13 - 15]. However, mutations in *NLGN3* and *NLGN4* have been recorded in neurodevelopmental disorders [7,16, 17].

The contributing role of NLGNs in autism has been extensively examined at the genetic level. El-Kordi et al. demonstrated that NLGN4 null mutant mice models showed high autism severity scores. The severity of autism was represented as reduced social interaction, ultrasonic communication, altered aggression, enhanced self-grooming and circling behavior [18], depression, anxiety and tic disorders [15]. Children with autism exhibit a significant cognitive and social impairments [19]. These impairments may be the most complex core challenge facing autistic patients [20]. The most common scales used for the assessment of the severity of autism and social impairment among autistic patients are childhood autism rating scale (CARS) and social responsiveness scale (SRS), respectively [21, 22].

The present study has been designed to evaluate NLGN4 level in plasma samples of autistic patients compared to the healthy matched controls in an attempt to ascertain whether NLGN4 could be considered as a biomarker for autism, and also to study the correlation between the plasma NLGN4 level and the degree of severity of autism and social impairment in autistic patients.
Materials and Methods

Participants

88 autistic children, aged between 3 and 12 years (5.5 ± 2.2) were recruited from Autism Research and Treatment Center, Faculty of Medicine, King Saud University, King Khalid University Hospital, Riyadh, Saudi Arabia. A clinical psychologist clinically diagnosed all autistic subjects, and the diagnosis was confirmed using the revised Autism Diagnostic Interview (ADI-R), the Autism Diagnostic Observation Schedule (ADOS) and Developmental, dimensional diagnostic interview (3DI). 33 age and sex-matched control participants, aged between 3 and 9 years (5.3 ± 1.2), were recruited from the pediatric clinic, King Khalid University Hospital, Riyadh, Saudi Arabia. Participants were excluded from the study if they had Fragile X, serious neurological disorders or any other medical conditions.

The severity of autism and the social impairment were determined using CARS and SRS, respectively [21, 22].

Sample Collection

Blood samples were drawn from 88 autistic children and 33 controls in the early morning after overnight fasting and immediately collected into tubes containing EDTA. Blood samples were centrifuged at 4 °C at 3000 g for 20 min. The plasma was decanted into different aliquots and stored at −80 °C until analysis.

Biochemical Analysis

Determination of Neuroligin 4

Plasma level of NLGN4 were measured using a commercial ELISA kit specific for NLGN4X (Cat. # E6841h) according to the manufacturer’s instructions (Wuhan EIAAb Science Inc., China). This immunoassay kit allows the in vitro quantitative determination of
human NLGN4 concentrations in plasma. The microtiter plate provided in this kit had been pre-coated with an antibody specific to NLGN4. Standards and samples were then added to the appropriate microtiter plate wells with a horseradish peroxidase (HRP)-conjugated antibody and incubated. Substrate solutions were then added to each well. The enzyme-substrate reaction was terminated by the addition of a sulfuric acid solution, and the color change was measured spectrophotometrically at 450 nm. The concentration of NLGN4 in the samples was then determined by comparing the optical density of the samples to the standard curve.

Statistical Analyses

The statistical package for social science (SPSS, Chicago, IL, USA, version 22) was used. In all statistical analyses, an independent t-test with \( p \leq 0.05 \) was considered as statistically significant. Statistical analysis was constructed in the present study to evaluate the plasma NLGN4 level among subjects diagnosed with autism in comparison to the controls. The correlation between levels of NLGN4 and the risk of autism, presented as CARS and SRS scores, was evaluated to determine if there was a threshold level of NLGN4 above which an increased risk of this disorder may appear. The receiver operating characterizing curve (ROC) was used to assess the specificity and sensitivity of NLGN4. ROC analysis was performed as a comprehensive way to measure the accuracy of the studied markers. The area under the curve (AUC) provides a useful metric to compare different biomarkers. AUC value close to 1 indicates an excellent diagnostic and predictive marker, whereas a curve that lies close to the diagonal (AUC = 0.5) has no diagnostic utility. AUC close to 1 is always accompanied by satisfactory values of specificity and sensitivity of the biomarker. Moreover, the predictiveness diagrams of the measured parameters were drawn in which the x-axis
represents the percentile rank of the biomarker, the y-axis represents the probability of identifying the disease, and the horizontal line is the prevalence of the disease.

**Results**

Plasma concentration of NLGN4 was compared between children with autism and their age-matched controls, as presented in Table 1. The results show a highly significant increase ($p = 0.001$) in plasma concentration of NLGN4 in autistic children in comparison with healthy controls. Moreover, the subjects with autism were classified according to their CARS, SRS scores and age into different subgroups of autism severity (mild-moderate autism or severe autism). We observed that the level of NLGN4 in subgroups of patients showing different levels of cognitive and social impairments (CARS, and SRS scores, respectively) was increased in the mild-moderate subgroup compared to the severe subgroup, but the difference did not reach any statistical significance. Additionally, no significant difference between NLGN4 level and age in subjects with autism was observed (Table 1). The individual distribution of data in autistic subjects compared to the controls is presented in figure 1.

Table 2 and Figure 2 demonstrate the receiver operating characteristics (ROC) analysis presented as the area under the curve (AUC), cutoff values, specificity and sensitivity of NLGN4 in autistic subjects and subgroups of patients. ROC curve analysis enables the best cutoff for clinical purposes. Sensitivity and specificity vary with the cutoff chosen for the test and depend upon the clinical context of researchers. This study indicated AUC around 0.751, which is considered as a satisfactory value of accuracy with high specificity and sensitivity (72.2%, 72.7%, respectively). The best cutoff value obtained from the ROC curve data analysis was 9.18 ng/ml. NLGN4 showed good specificity and sensitivity but not good
enough for clinical purposes. The table also demonstrates that NLGN4 in the subgroup of patients exhibited AUC value close to 0.5, which is considered as poor.

**Discussion**

The present study is the first to determine the plasma level of NLGN4 in autism; all previous studies were done at the genetic level. This study shows a significantly increased level of the plasma NLGN4 in autistic subjects compared to matched controls, suggesting that NLGN4 may play a role in the pathogenesis of autism. Furthermore, ROC curve results confirmed that NLGN4 may be considered for use as a risk biomarker for autism.

Although most of the previous experimental studies on nervous system disease biomarkers were done on tissues or whole blood samples, the study of their level in plasma as a complex body fluid can reflect the pathophysiological activity in the CNS. Since 500 ml of CSF in the human body is absorbed into the blood every day, this makes plasma a good source of biomarkers of nervous system diseases [23].

Genetic studies of autism have revealed various numbers of gene mutations in synaptic proteins that are implicated in autism and lead to synaptic dysfunction and cause wide range of behavioral impairments and social interaction deficits [15, 24-26].

NLGN4 is known to play a critical role in synaptic function, formation and stabilization, so the impaired NLGNs may lead to excitatory/inhibitory imbalance and selective loss of inhibitory function of neurons [9, 27, 28]. In addition, the relationship between glutamate, GABA and the neuroligin family has been established in autism. It has been shown that neuroligins are localized at both glutamatergic and GABAergic synapses in hippocampal neurons. So, alteration in glutamate and GABA levels in autistic samples have been shown to
affect neuroligin function in synapse stabilization [28, 29]. Taken together, the higher level of NLGN4 reported in the present study may be a consequence of synaptic dysfunction associated with autism, or due to alteration of the other components involved in synaptogenesis. However, these data should be treated with caution until further studies with a larger sample size are performed to validate these results and to elucidate the mechanism by which expression of NLGN4 may contribute to the pathogenesis of autism.

Chih et al. showed that a nonsense mutation in the NLGN4 in hippocampal neurons caused retention of NLGN4 protein in the endoplasmic reticulum. They also concluded that mutant NLGN4 protein failed to promote presynaptic differentiation, and hence leads to complete functional inactivation [30]. Another study carried out by Talebizadeh et al. suggested that genetic alteration other than mutations such as splice variants may lead to abnormal function of NLGN4 in autism spectrum disorder [17]. These findings may explain the higher plasma level of NLGN4 observed in the present study, suggesting that mutations may lead to increased production of NLGN4 but without any functional effect.

Although there was no correlation between NLGN4, CARS and SRS observed among the subgroups of autistic subjects in the present study, it seems that NLGN4 alteration may affect the cognitive and social behaviors in subgroups of patients [14]. This could be due to inadequate sample size that failed to detect significant changes between NLGN4 and the behavioral scores measured.

One of the limitations of the current study is the small sample size. The lack of correlation between plasma levels of NLGN4 and cognitive problems or social impairment could be attributed to the sample size, as the statistical power failed to detect significant changes between the studied parameters. A further potential limitation is the lack of previous
research on plasma NLGN4; we were unable to assess the effect of NLGN4 on the severity of autism. Therefore, there is a need for similar studies on larger sample sizes using plasma to fill this gap in the literature and to understand the potential links between NLGN4 and cognition and social impairment.

Conclusion

This study highlights the possible relevance of plasma NLGN4 in the etiology of autism. ROC curve suggests that NLGN4 could be used as a risk biomarker for autism. Future studies ought to focus in the exact role of NLGN4 in the pathophysiology of autism. Furthermore, interventional studies are recommended to explore the potential links between NLGN4 and features of autism.

Acknowledgment

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Statement of Ethics

This study was approved by the Medical Ethics Committee of King Saud University, according to the most recent declaration of Helsinki principles. Parents of children provided informed consent prior to participation in the study according to the guidelines of the Ethics Committee of King Saud University, King Khalid University Hospital.

Disclosure Statement

The authors declare that they have no conflicts of interest.
Author Contributions

Laila Y. Al-Ayadhi designed the study, conducted the study and co-drafted the manuscript. Hanan Y. Qasem performed data analysis and drafted the manuscript. Hend Al-Ghamdi carried out the biochemical assays and literature review. Nadra E. Elamin co-drafted and revised the manuscript. All authors have read and approved the final manuscript.

References


Table 1: Plasma neuroligin 4 levels (ng/ml) control and autistic groups, CARS, SRS, and age in subjects with autism

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>N</th>
<th>Mean</th>
<th>STD Deviation</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLGN4 (ng/ml)</td>
<td>Control</td>
<td>33</td>
<td>6.821</td>
<td>5.521</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Autism</td>
<td>88</td>
<td>12.299</td>
<td>5.348</td>
<td></td>
</tr>
<tr>
<td>CARS</td>
<td>Mild to moderate</td>
<td>53</td>
<td>12.881</td>
<td>5.091</td>
<td>0.271</td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>35</td>
<td>11.417</td>
<td>5.676</td>
<td></td>
</tr>
<tr>
<td>SRS</td>
<td>Mild to moderate</td>
<td>22</td>
<td>13.469</td>
<td>4.839</td>
<td>0.213</td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>66</td>
<td>11.909</td>
<td>5.486</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>Age ≤ 6 years</td>
<td>66</td>
<td>12.540</td>
<td>5.221</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Age &gt; 6 years</td>
<td>22</td>
<td>11.575</td>
<td>5.777</td>
<td>0.492</td>
</tr>
</tbody>
</table>

NLGN4-neuroligin 4; CARS-Childhood Autism Rating Scale; SRS-Social Responsiveness Scale
Table 2: ROC analysis data of NLGN4 in the autistic group, CARS, SRS, and age in autistic children

<table>
<thead>
<tr>
<th></th>
<th>AUC</th>
<th>Best cutoff value</th>
<th>Sensitivity%</th>
<th>Specificity%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with autism</td>
<td>0.751</td>
<td>9.18</td>
<td>72.7</td>
<td>72.2</td>
</tr>
<tr>
<td>CARS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild to moderate</td>
<td>0.570</td>
<td>13.034</td>
<td>58.5</td>
<td>54.3</td>
</tr>
<tr>
<td>Severe</td>
<td>0.430</td>
<td>13.034</td>
<td>45.7</td>
<td>41.5</td>
</tr>
<tr>
<td>SRS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild to moderate</td>
<td>0.573</td>
<td>13.49</td>
<td>59.1</td>
<td>51.5</td>
</tr>
<tr>
<td>Severe</td>
<td>0.427</td>
<td>13.66</td>
<td>47</td>
<td>40.9</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 6 years</td>
<td>0.544</td>
<td>12.513</td>
<td>56.1</td>
<td>45.5</td>
</tr>
<tr>
<td>&gt; 6 years</td>
<td>0.456</td>
<td>13.034</td>
<td>0.455</td>
<td>0.439</td>
</tr>
</tbody>
</table>

AUC- Area Under Curve; CARS-Childhood Autism Rating Scale; SRS-Social Responsiveness Scale; ROC-Receiver Operating Curve; NLGN4-Neuroligin 4
Figure 1: Normal distribution of NLGN4 in A) controls and B) subjects with autism groups. NLGN4-Neuroligin 4
**Figure 2:** ROC curve of NLGN4 (ng/ml) in autistic groups

ROC - Receiver Operating Curve; NLGN4 - Neuriligin 4