THE COMBINED ROLE OF SEROTONIN AND INTERLEUKIN-6 AS BIOMARKER FOR AUTISM

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Abstract—Autism is a severe neurodevelopmental disorder characterized by impairments in social interaction and repetitive behaviors. Diagnosis of autism is currently phenotype based with no reliable laboratory test available to assist clinicians. It has been shown that dysfunction of serotonin (5-HT) and interleukin-6 (IL-6) are involved in autism. The goal of this study was to evaluate the combined role of 5-HT and IL-6 as potential biomarkers for autism. The whole blood concentration of 5-HT and plasma concentration of IL-6 of individuals with autism were significantly elevated compared with the control group, and the concentration of 5-HT and IL-6 had positive correlations with the severity of autism. The results of receiver operating characteristic (ROC) analysis indicated that the combination of 5-HT and IL-6 produced the best sensitivity and specificity for diagnosis of autism. Therefore, the present study has revealed a simple clinical method with great potential for assisting the diagnosis of autism. © 2014 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: autism, serotonin (5-HT), interleukin-6 (IL-6), biomarker.

INTRODUCTION

Autism is a severe neurodevelopmental disorder of childhood typically characterized by substantial impairments in social skills and communications, restricted interests, and repetitive behaviors, whose symptoms manifest in the early developmental period (Association, 2013). A recent systematic review of literature suggested a median Autism Spectrum Disorder (ASD) prevalence of 62/10,000 globally and 65.5/10,000 in the US and Canada (Elsabbagh et al., 2012). The Centers for Disease Control (CDC) reported an estimate of 1 in 88 children identified as having ASD in the United States (Baio, 2012). However, it has been noted that the incidence showed a significant increase in recent years (Hertz-Picciotto and Delwiche, 2009).

There are diagnostic criteria listed in the Diagnostic and Statistical Manual of Mental Disorders (DSM) and the International Statistical Classification of Diseases and Related Health Problems (ICD-10). Assessment tools such as the Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview-Revised (ADI-R) are widely used. However, the disorder is currently diagnosed solely using core behavioral criteria selected to define autism, and there is presently no trusted laboratory test available to aid clinicians. In addition, individuals with autism vary enormously in clinical presentation, severity, developmental trajectory, and treatment response. This complexity is spurring an intensive search to identify biological markers that are able to aid clinicians in achieving earlier diagnoses and in predicting clinical prognosis as well as treatment response.

Several lines of evidences suggested that alterations in serotonergic neurotransmitter system might represent one of the biological substrates of the disorder. Serotonin (5-hydroxytryptamine; 5-HT) has specific functions in the central nervous system (CNS) and periphery where it regulates many physiological activities. In human studies, 5-HT has been demonstrated to be important for prenatal and postnatal brain development by regulating both, serotonergic outgrowth and maturation of target regions (Whitaker-Azmitia, 2001). Problems in serotonergic signaling in some of these systems have been implicated as comorbidities that occur with autism (Meyer, 2013). Hyperserotonemia, or elevated blood 5-HT levels are occurred in 25–35% of individuals with ASD (Hanley et al., 1977; Cook et al., 1993; Hranilovic et al., 2007). Some data suggested an association of hyperserotonemia with stereotyped or self-injurious behavior, although results have been inconsistent (Kolevzon et al., 2010; Sacco et al., 2010; Veenstra-VanderWeele et al., 2012). Abnormalities in the brain serotonin system are also reported in ASD, including evidence of altered serotonin synthesis and receptor binding, as well as dystrophic
serotonergic axons (Chugani et al., 1997; Makkonen et al., 2008; Nakamura et al., 2010; Azmitia et al., 2011). Thus, the overall evidence implicates the dysfunction of 5-HT system in autism.

Growing bodies of evidences implicate immunological disturbances in autism and a lot of studies have reported cytokine abnormalities in the peripheral blood of autistic patients (Ashwood et al., 2011; Goines and Ashwood, 2013; Ricci et al., 2013). Observations indicated significant increases in the plasma level of interleukin-6 (IL-6) in autism compared with typically developing controls (Emanuele et al., 2010; Ashwood et al., 2011; Malik et al., 2011). Further, increased IL-6 is found in postmortem brain specimens from autism subjects (Li et al., 2009; Wei et al., 2011). What is more, Vargas et al. have demonstrated that IL-6 was increased in the anterior cingulated gyrus of autistic brains and also in the cerebrospinal fluid of autistic children (Vargas et al., 2005). IL-6 levels are associated with core deficits of autism or impairments in associated behaviors and/or onset patterns of autism (Okada et al., 2007; Ashwood et al., 2008).

It has been demonstrated that IL-6 is an important target of 5-HT in vivo (Miyata et al., 2001; Tian et al., 2011; Li et al., 2013). Evidence suggested that 5-HT7 receptor stimulation in human microglial cells is linked to induction of IL-6 gene expression (Mahe et al., 2005). The 5-HT receptor antagonist rescues IL-6-induced pulmonary hypertension (Miyata et al., 2001). The separate role of blood 5-HT and IL-6 in autism, coupled with the evidence for interaction between them led us to examine the role of peripheral 5-HT and IL-6 as biomarkers in autism.

Therefore, the present study aimed to evaluate the alterations of 5-HT and IL-6 levels in autism and their roles as potential biomarkers for autism. Importantly, the present study sought to examine the combined effects of 5-HT and IL-6 as biomarker in autism, which may supply a more useful and reliable method assisting the diagnosis of autism.

**EXPERIMENTAL PROCEDURES**

**Subjects**

Thirty-five individuals with autism and thirty-one healthy control individuals were selected after clinical evaluations. Participants were placed in one of two groups: (1) diagnosed with autism or (2) confirmed as typically developing controls. Autism diagnosis was performed using gold-standard assessments based on Diagnostic and Statistical Manual of Mental Disorders (4th edition) (DSM-IV) criteria by qualified trained clinicians. Participants were excluded from the study if they had a diagnosis of fragile X syndrome, epileptic seizures, obsessive–compulsive disorder, affective disorders, or any additional psychiatric condition. Also excluded were those with inflammation, known endocrine, cardiovascular, pulmonary, liver, kidney or neurological diseases. The control individuals were normally developing, healthy individuals, unrelated to the autistic subjects and without any of the exclusion criteria. Two groups of individuals were matched on age and gender, and they were not taking any medication that could interfere with inflammation four weeks prior to the screening and in good health at time of blood draw. The intelligence quotient (IQ) was based on the previous recording in the hospital. Two individuals with autism were removed from the study due to refuse to phlebotomize. Thus, the participants consisted of thirty-three individuals with autism and thirty-one typically developing individuals.

An informed consent was obtained from the parents of each participant case prior to inclusion in the study. The ethics committee of East China Normal University approved this study.

**Behavioral assessment**

The Childhood Autism Rating Scale (CARS) score was completed as a measurement of the severity of autism. CARS consists of 15 domains (relating to people; emotional response; imitation; body use; object use; listening response; fear or nervousness; verbal communication; non-verbal communication; activity level; level and reliability of intellectual response; adaptation to change; visual response; taste, smell and touch response; and general impressions). Each domain is scored on a scale ranging from one to four, with higher scores associated with a higher level of impairment. An individual with a CARS score $\geq 30$ is considered to have autism (Schopler et al., 1980).

**Biochemical analysis**

After overnight fast, two tubes of blood samples (5 mL/tube) were collected from each subject in both groups in acid-citrate dextrose Vacutainers (BD Biosciences; San Jose, CA, USA) between 08:00 and 09:00. One tube was measured by high performance liquid chromatography (HPLC) with fluorometric detection for whole blood 5-HT (WB-5-HT) as described previously (Anderson et al., 1981). Intra-assay and interassay coefficients of variation were 2.0 and 5.8%, respectively. Another tube was centrifuged at 3500 rpm at 4°C for 15 min. Plasma were obtained and deep frozen (at $-80\,^\circ\text{C}$) until analysis time. The IL-6 in plasma was measured by using the quantitative sandwich enzyme immunoassays with enzyme-linked immunosorbent assay kits (R&D Systems Europe Ltd., Abingdon, Oxon, UK) following the manufacturer's instructions. The detection limits for IL-6 were 2.0 pg/mL. Intra-assay and interassay coefficients of variation were 4.5 and 7.2%, respectively.

**Statistical analysis**

Results were performed using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). Group comparisons to categorical variables were performed by using Pearson’s chi-square test or Fisher’s exact test, and to continuous variables were performed by Student’s $t$-test for normally distributed data and the Wilcoxon rank-sum test for non-normally distributed data. Receiver operating characteristic (ROC) analysis was performed to test the specificity and sensitivity of the biomarkers for
detecting autism as described in previous papers (Gallop et al., 2003; Pepe et al., 2006). In summary, logistic regression models were estimated for each marker individually and combination of two markers to differentiate between individuals with autism and normal healthy controls. ROC curves were constructed for each logistic regression model and the area under the curve (ROC-AUC) was compared between each markers or marker combination using a non-parametric method (DeLong et al., 1988). Spearman rank order correlations analysis was used to determine the relationship between CARS scores, 5-HT and IL-6. In all statistical analyses, \( p < 0.05 \) was used as the criteria for statistical significance.

**RESULTS**

**Demographic features**

Demographic features between two groups were similar. There were no statistically significant differences between the groups with respect to the differentiations of age, sex, body mass index (BMI) (Table 1).

**The production of WB 5-HT and plasma IL-6**

There was a significant difference noted in median whole blood concentration of 5-HT and plasma concentration of IL-6 between the autism and control individuals. The individuals with autism showed an elevated median 5-HT concentration of 158.96 ng/mL (range, 67.44–336.27 ng/mL), whereas the control individuals exhibited a median concentration of only 85.67 ng/mL (range, 15.29–173.26 ng/mL), \( p < 0.001 \) (Fig. 1A). The individuals with autism showed an elevated median IL-6 concentration of 166.49 pg/mL (range, 75.86–333.38 pg/mL), whereas the control individuals exhibited a median concentration of only 97.98 pg/mL (range, 52.13–167.59 pg/mL), \( p < 0.001 \) (Fig. 1B).

**ROC curve analysis**

To assess the usefulness of these biomarkers as adjunct in the diagnosis of autism, an ROC analysis was performed (Fig. 2). The optimal cut-off point for using 5-HT as a biomarker for autism was 111.20 ng/mL. This cut-off point was associated with a sensitivity of 78.79% and a specificity of 80.65%. \( (\text{AUC} = 0.86; 95\% \text{ confidence interval } (\text{CI}), 0.75–0.94, \ p < 0.0001) \). The optimal cut-off point for using IL-6 as a biomarker for autism was 103.98 pg/mL. This cut-off point was associated with a sensitivity of 87.88% and a specificity of 74.19%. \( (\text{AUC} = 0.85; 95\% \text{ CI}, 0.74–0.93, \ p < 0.0001) \). The combination of two factors had a sensitivity of 84.85% and a specificity of 96.77% \( (\text{AUC} = 0.96; 95\% \text{ CI}, 0.88–0.99, \ p < 0.0001) \). (Table 2)

In addition, the diagnostic ability was significantly improved by the combination of two factors when compared with WB 5-HT \( (P = 0.009) \) and plasma IL-6 \( (P = 0.018) \), however, no differences were identified between WB 5-HT and plasma IL-6 \( (P = 0.903) \).

**Correlations**

For autistic individuals, the relationship between the levels of 5-HT, IL-6 and the level of autism measured by the CARS scores was also evaluated. There were positive correlations between 5-HT and CARS scores \( (R = 0.63, \ p < 0.001) \), IL-6 and CARS scores \( (R = 0.63, \ p < 0.001) \), 5-HT and IL-6 \( (R = 0.87, \ p < 0.001) \) (Fig. 3).

**DISCUSSION**

The results of this study demonstrated that as compared to the healthy controls, the individuals with autism showed a higher level of 5-HT and IL-6. And ROC analysis indicated the 5-HT and IL-6 were the potential biomarkers in diagnosis of autism. The combination of two factors gave the best sensitivity and specificity in diagnosis of autism. The WB 5-HT and plasma IL-6 are associated with the severity level of autism measured by the CARS scores.

In the present research, individuals with autism exhibited elevated WB 5-HT concentrations, which is consistent with previous finding of other autism studies (Hanley et al., 1977; Cook et al., 1993; Betancur et al., 2002; Janusonis, 2008; McNamara et al., 2008; Mostafa and Al-Ayadhi, 2011). Approximately 95% of the body’s serotonin is found in the bowel (Gershon et al., 1965), and 99% of the serotonin in the bloodstream is located in blood platelets, which takes up overflow serotonin from the gut. Hyperserotonemia is considered to be the most commonly observed and well-replicated change in autism. Elevated 5-HT blood levels are seemingly autism specific, as they are not present in cognitively impaired individuals (Mulder et al., 2004). Studies showed in utero exposure to drugs that raise blood serotonin levels, including cocaine and alcohol, increase rates of autism in children (Davis et al., 1992; Nanson, 1992). In human studies, 5-HT has been demonstrated to be important for prenatal and postnatal brain development (Whitaker-Azmitia, 2001). The well-replicated plateau hyperserotonemia of autism still remains unexplained. Possible explanations for the increased exposure of the platelet to serotonin may be due to the alteration in the platelet’s handling of

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Table 1. Clinical and demographic features of autistic and control subjects

<table>
<thead>
<tr>
<th></th>
<th>Autism (n = 33)</th>
<th>Control (n = 31)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Age (mean ± SD)</td>
<td>12.21 ± 2.67</td>
<td>12.52 ± 2.14</td>
<td>0.618</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
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<tr>
<td>Male</td>
<td>27</td>
<td>24</td>
<td>0.662</td>
</tr>
<tr>
<td>Female</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>22.61 ± 4.87</td>
<td>23.79 ± 5.09</td>
<td>0.348</td>
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<tr>
<td>IQ</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MiR</td>
<td>13</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>MoR</td>
<td>15</td>
<td>0</td>
<td>–</td>
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<tr>
<td>SeR</td>
<td>5</td>
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\( N \), number of subjects; BMI, body mass index; IQ, intelligence quotient; MiR, mild mental retardation (IQ between 50 and 70); MoR, moderate mental retardation (IQ between 30 and 50); SeR, severe mental retardation (IQ < 30).
serotonin (Anderson et al., 2012). In autistic children, serotonin levels remain elevated up to age 16, whereas in normal individuals they peak at around age 6 and fall thereafter (Leboyer et al., 1999). This led to our decision of including the individuals above 6 years in the present study.

Recent studies have reported an association of cytokines with autism. IL-6 was originally found to be a major inducer of immune and inflammatory response (Rose-John et al., 2006). In the present study, individuals with autism displayed elevated plasma IL-6 concentrations as compared with control individuals, which is consistent with previous studies of autism (Ashwood et al., 2011; El-Ansary et al., 2011; Ricci et al., 2013). Also, IL-6 was significantly increased in the brains of ASD patients as compared with the controls (Vargas et al., 2005; Li et al., 2009; Wei et al., 2011). IL-6 is elevated in the cerebral spinal fluid and brain homogenates in the presence of brain injury or inflammation (Van Wagoner and Benveniste, 1999). Elevated IL-6 in the autistic brain could alter neural cell adhesion, migration and also cause an imbalance of excitatory and inhibitory circuits (Wei et al., 2011).

The positive correlation between 5-HT and IL-6 levels in the human data can be interpreted in a number of ways, as this relationship could be mediated at multiple levels. 5-HT is able to regulate IL-6 secretion through the activation of the 5-HT2B receptor and the ERK1/2 pathway (Li et al., 2013). Levels of numerous cytokines in the brain are enhanced by treatment with serotonin-specific reuptake inhibitor (SSRIs), and blocked by non-steroidal antiinflammatory drugs (NSAIDs) (Snyder, 2011). The investigators have linked these biochemical findings to behavior (Warner-Schmidt et al., 2011). Our research also confirmed that 5-HT and IL-6 were associated with severity of autism measured by CARS.

A biomarker can be defined as a biological variable associated with the disease of interest across and within individuals, measurable directly in a given patient or in his/her biomaterials using sensitive and reliable quantitative procedures (Gabriele et al., 2014). Although some researchers regarded 5-HT (Hammock et al., 2012) and cytokines (Abdallah et al., 2013) as a potential biomarker for autism, only association analysis was used in most of studies. However, it is not sufficient to define the biomarker only by correlation analysis. In the present research, ROC analysis was performed to assess the usefulness of these biomarkers. We found that, in comparison with singular effect of 5-HT and IL-6, the combination of two factors produced the best sensitivity and specificity.
specificity in diagnosis of autism, and the AUC is 0.96. The AUC provides a useful metric to compare different biomarkers. Whereas an AUC value close to 1 indicates an excellent diagonal and predictive marker, a curve that lies close to the diagonal (AUC = 0.5) has no diagnostic utility. AUC close to 1.00 is always accompanied by satisfactory values of specificity and sensitivity of the biomarker. High sensitivity means that in most cases autism will be identified, while high specificity means few individuals whose positive test results on the autism prediction were false. This shows their usefulness as predictive biomarkers. This could be supported by the high sensitivity and specificity recorded through ROC analysis. To our knowledge, this is the first study to use the combined ROC to analyze the potential biomarker for autism.

The present study has some limitations. Firstly, diagnostic procedures applied in USA/Europe using the Autism Diagnostic Interview-Revised and Autism Diagnostic Observation Schedule Generic were not used in the diagnostic process in China. This shortcoming was met by long clinical experience by the psychiatrist who was aware of the core behaviors in autism stated by the American Academy of Pediatrics in its Embargo from 2007 (Johnson and Myers, 2007). Secondly, this study used a quite small sample, so the findings require replication in future research using larger samples from multicenters. Thirdly, mechanistic studies on animal model of autism are also important. It is possible that a closer analysis of the deep relations of 5-HT, IL-6 and autism will offer insights into novel pharmacologic treatments.

CONCLUSIONS

The present study showed that, as compared to healthy individuals, individuals with autism showed an elevated level of 5-HT and IL-6. 5-HT and IL-6 showed a significant positive correlation with the severity of autism. The combination of 5-HT and IL-6 performed the best as a biomarker in the diagnosis of autism.

CONFLICT OF INTEREST

No potential conflict of interests relevant to this article was reported.

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