

Original Article

Amyloid precursor protein associates with autism spectrum disorder: a potential candidate biomarker for early screening

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Abstract: There is presently no clinical laboratory test for diagnosing autism. We aim to investigate plasma secreted amyloid precursor protein alpha (sAPP- α) as a possible peripheral biomarker in the diagnosis of autism in 116 Han children using case-control study. A sensitive enzyme-linked immunosorbent assay (ELISA) method was used to detect plasma sAPP- α and brain derived neurotrophic factor (BDNF). A self-designed questionnaire was used to screen impact factors of sAPP- α . Compared to control children, there were significantly increased levels of sAPP- α in the known autistic children ($P < 0.0001$) while BDNF showed no difference ($P > 0.05$). In addition, in a subset of patients with severe autism, plasma level of sAPP- α was significantly elevated compared to controls and patients with mild-to-moderate autism. There was a significant positive correlation between sAPP- α level and the parental childbearing age, and negative correlations between sAPP- α and birth weight, as well as between sAPP- α and gestational weeks. Additionally, elevated sAPP- α was found in premature, maternal infection, neonatal hyperbilirubinemia and neonatal hypoxic ischemic encephalopathy ($P < 0.05$, respectively). Our findings support the use of sAPP- α as an accurate and sensitive laboratory parameter for early screening of autism spectrum disorders (ASD). Elder parents' age at childbearing, low birth weight, premature, maternal infection, neonatal hyperbilirubinemia and neonatal hypoxic ischemic encephalopathy may be responsible for the increase in sAPP- α .

Keywords: Autism spectrum disorders (ASD), amyloid precursor protein (APP), brain derived neurotrophic factor (BDNF)

Introduction

Autism, or newly defined as autistic spectrum disorders (ASD), is a neurodevelopmental disorder. It is mostly characterized by deficits in verbal communication, impairment in social interactions, and the presence of repetitive, stereotyped, and compulsive behaviors, according to Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-V) by the American Psychiatric Association [1]. About 1 in 68 children has been identified with ASD according to estimates from CDC's Autism and Developmental Disabilities Monitoring (ADDM) Network.

Although there are diagnostic criteria listed in DSM-V, the International Statistical Classifica-

tion of Diseases and Related Health Problems (ICD-10), the Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview-Revised (ADI-R), the disorder is currently diagnosed solely using core behavior criteria selected to define autism typically during the toddler or preschool years at the earliest [2-4]. There is presently no clinical laboratory test for diagnosing autism [5]. To begin intervention at the earliest possible time, it is necessary to develop biological quantitative methods to predict the presence or risk of autism [6]. Since the etiology of the vast majority of cases of ASD is unknown, the biochemical indicators have proven to be incredibly complex [7].

In this study we measured level of plasma secreted amyloid precursor protein alpha

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Table 1. Comparison of sAPP- α (ng/ml) and BDNF (ng/ml) between ASD group and control group

	ASD (n)	Control (n)	t	P
sAPP- α	47.41 \pm 23.91 (116)	18.42 \pm 9.9 (116)	7.91	<0.0001
BDNF	9.97 \pm 4.60 (79)	11.04 \pm 5.44 (65)	1.28	0.2028

Table 2. Comparison of sAPP- α (ng/ml) in different subgroup children

Group	n	Mean	SD
Control	116	18.42	9.90
Mild-to-moderate	91	42.76	19.87
Severe	25	64.44	16.95

F=17.62, P<0.001.

(sAPP- α) in 116 children and found that sAPP- α could be used as a possible peripheral biomarker for early screening of ASD.

Materials and methods

Sample demographics

Blood samples were acquired from 116 children diagnosed as autism with DSM-V criteria at the Hubei Maternal and Children's Hospital (Wuhan, China) between January 2012 and December 2014. The patients consisted of 99 males and 17 females (ratio 5.82:1), age from 28-45 months (35.05 \pm 8.21 months). The patients with confirmed autism diagnoses were further classified as mild-to-moderate (score 30-36, n=91) and severe (score \geq 37, n=25) disease, as specified by the Childhood Autism Rating Scale (CARS). Additionally, gender-, age-, and domicile-matched normal controls (n=116) were acquired from the same source. Medication history and other clinical features for most patients were non-significant. The exclusion criteria for all subjects included the presence of Rett syndrome, Asperger syndrome, fragile X syndrome, and other neurological disorders, inherited metabolic disorders and epilepsy. Written informed consent was obtained from at least one parent. The study was approved by the Ethics Committee of Hubei Maternal and Children.

Detection of sAPP- α and brain derived neurotrophic factor (BDNF) by ELISA

sAPP- α and BDNF were detected by commercially available ELISA kits (IBL, Hamburg,

Germany, the sensitivity is 0.09 ng/ml) according to the manufacturer's instructions. In order to eliminate the experimental error and to keep the data credibility, we used the same batch

of reagent and keep the experimental group and the control group in the same plate. The optical density (OD) values were measured with Model 680 Series Microplate Reader (Bio-Rad, USA). The average values were got after three times repeat.

Self-administered questionnaires

We developed a self-made original questionnaire including three parts (general situation, pregnancy and perinatal situation), 15 items in total. It was first pre-tested on a group of children in the Child Health Care Clinic at Hubei Maternal and Children's Hospital and appropriate changes were made to improve the comprehensibility. Cronbach's coefficient was found to be 0.75, which showed an internal reliability of the questionnaire. Content Validity Ratio (CVR) was calculated and found to be acceptable. The questionnaires were completed by parents with qualified senior staff present to provide clarifications. All investigators had completed strict professional training with unified standards before the investigation was initiated. No data were collected until written informed consent was received from either parent. The self-administered questionnaires were collected after completion and phone interviews were conducted for missing items to ensure reliability and completeness of the information acquired. For data quality control, 5% of the study parents were randomly selected and asked to re-complete 15 questions from the questionnaires. On average, 14.3 questions per person were answered consistently as previous answer, confirming a 95% concordance rate.

Statistics

The data were analyzed using SAS version 8.2. The results are expressed as mean \pm SD. Two-tailed t-test, χ^2 test, variance analysis and multiple linear regression analysis were used. The threshold was set to 0.05 for determining statistical significance in all cases.

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Table 3. Comparison of impact factors between case and control

Variables	ASD ($\bar{x} \pm SD$)	Control ($\bar{x} \pm SD$)	<i>t</i>	<i>P</i>
Maternal age (y)	42.12 \pm 4.37	32.19 \pm 5.63	8.32	0.0043
Father age (y)	45.61 \pm 5.67	33.01 \pm 7.21	9.14	0.0031
Birth weight (kg)	2.15 \pm 0.78	3.16 \pm 0.51	4.15	0.0292
Gestational weeks	33.23 \pm 4.01	37.25 \pm 2.15	3.96	0.0391

Table 4. Comparison of incidence of jaundice, HIE and infection between ASD and control group

Variables	ASD N (%)	Control N (%)	χ^2	<i>P</i>
Pathological jaundice	97 (83.62)	18 (15.517)	21.37	<.0001
HIE	72 (62.06)	8 (6.89)	16.54	<.0001
Infection	88 (75.86)	12 (10.34)	18.11	<.0001

Table 5. Multivariate linear regression analysis of sAPP- α

Variables	Parameter Estimate	Standard Error	<i>t</i>	Standardized Pr> <i>t</i>	Estimate
Maternal age (y)	20.67	5.63	3.67	0.0003	0.17
Father age (y)	28.78	8.59	3.35	0.0010	0.15
Birth weight (kg)	-1.46	0.12	-11.44	<.0001	-0.63
Gestational weeks	-1.51	0.14	-10.71	<.0001	-0.58
Pathological jaundice	21.86	6.52	5.23	0.0001	0.15
HIE	27.45	9.26	6.51	0.0001	0.13
Infection	29.57	10.81	7.04	0.0001	0.10

Results

The autistic children have a higher level of sAPP- α compared to control children

We found a higher level of sAPP- α in plasma of the autistic children compared to control children but there is no significant difference in BDNF between the two groups (**Table 1**).

Plasma sAPP- α - increases in severe ASD children compared to mild-to-moderate children

Among the autistic children, severe grade children showed a higher plasma sAPP- α than that in mild-to-moderate patients ($P < 0.001$, **Table 2**).

Comparison of parental age, birth weight and gestational age between patients and control

The age of the parents in the ASD group was higher than that in the control group ($P < 0.001$), while the birth weight and gestational age were

less than those of the control group (**Table 3**).

Comparison of incidence of jaundice, hypoxic ischemic encephalopathy (HIE) and infection between ASD and control

The incidence of jaundice, HIE and infection in the ASD group were higher than those in the control group ($P < 0.001$, **Table 4**).

Multivariate linear regression analysis of sAPP- α

Multiple linear regression analysis showed that parental advanced age (more than 35 years), premature delivery, infection during pregnancy, pathological jaundice, HIE, born weight <2.0 kg were factors that would increase sAPP- α . Father's and mother's age might have positive correlations with sAPP- α , while birth weight and gestational age have negative correlation with sAPP- α (**Table 5**).

Discussion

The prevalence of ASD disorder has increased in recent years, likely due to a combination of increased social awareness, broader disease classification, and a real increase in disorder prevalence [8, 9]. Unfortunately, the neurobiological basis of the disorder is very poorly understood presently. Despite the fact that there are many hypotheses for the etiology and pathogenic mechanisms of this disorder, including genetics, environmental factors, perinatal high-risk factors, and vaccinations, there is no definitive one that could be defined to associate with the majority of autism [10].

The prognosis of ASD is poor and nearly half of the children with autism are not able to live independently, laying a heavy burden to the family and our society. Because of the significant increase in the prevalence of ASD and its heavy mental and economic burden on families and society, prevention and treatment of autism is currently becoming a major social health issue [11]. Only with early detection and timely intervention or treatment, would the

prognosis of autism be significantly improved [12]. Currently, the early diagnosis of autism solely depends on use of core behavior standards, especially early childhood and some of the behavior of the school age. There is no clinical laboratory testing method for diagnosis of autism. In order to give early intervenes in the early stages, it is necessary to investigate the use of biological quantitative methods to predict the occurrence or the risk of autism.

A common sign of autism is abnormal brain overgrowth, which may be used in the future as a quantitative diagnostic tool [13]. Brain overgrowth may be detected by signs of a central nervous system (CNS) anabolic state in 15% to 35% ASD children [14]. About 90% of children experience a certain period of degree of abnormal brain growth in early life [15]. Studies using magnetic resonance imaging (MRI) and head circumference measurements found brain growth in autistic children with abnormal regulation, which results in early overgrowth followed by abnormally slowed growth, occurs like the performance that brain overgrowth early in life but later slowing down [16]. In addition, some studies showed that the increased volume of brain white matter positively correlated to the impairment of the motor skills in autistic children [17, 18].

Taking into account of previous studies showing that brain-derived neurotrophic growth factor (BDNF) is associated with macrocephaly in autistic patients [19], we initially considered that BDNF might be likely a biomarker candidate. Nelson [20] reported that the levels of BDNF in the blood samples of the newborns with autism were significantly higher than those in the control group. The results were further confirmed by Miyazaki's study [21] in which the levels of BDNF in cerebrospinal fluid and plasma were higher than those in control. These studies showed that BDNF levels in children with autism are abnormal, and suggested that the biological indicators may play a role in the development of autism and may become a potential tool for the diagnosis of autism and neural developmental disorder. Zhou et al. [22] utilized transmission disequilibrium test (TDT) to analyze the relationship between BDNF gene dinucleotide repeat polymorphism and autism. They found a transmission disequilibrium of BDNF gene dinucleotide repeat polymor-

phism in severe autistic children. Thus severe children with autism may be potentially related to the BDNF gene polymorphism sites. The candidate minor gene of severe ASD may be BDNF gene, or locates in the vicinity of the dinucleotide repeat polymorphism. However, there are contrary findings. Philippe [23] studied 38 autism high risk families using TDT and found no significant association between BDNF and ASD. However, after further searching literature, we discovered that not only is BDNF unreliable, but it is also not mutually exclusive to autism patients [24]. In summary, the discovery of secreted APP- α elevated in children with autism led to our speculation that BDNF might not be the specific or potential candidate for early screening of ASD. Indeed this has been suggested by studies of APP, a type I transmembrane protein that undergoes proteolytic processing by secretase enzymes to liberate soluble fragments. APP expression is regulated at both the transcriptional and post-transcriptional level.

Proteolytic cleavage of APP by the sequential actions of β - and γ -secretase form the neurotoxic amyloid beta (A β) peptide, which typically consists of 40 or 42 amino acid residues (the amyloidogenic pathway) [25]. On the other hand the non-amyloidogenic pathway [26] consists of APP cleavage by α -secretase which yields the neurotrophic product, secreted APP- α , APP- β . As α -secretase cleaves APP within the A sequence, A β formation is subsequently prevented. Taken together our results demonstrate that the non-amyloidogenic pathway may be preferentially active in severely autistic patients. These findings apparently do not extend to patients with mild-to-moderate autism, providing a biochemical correlate of phenotypic severity.

In 2006, Sokol and colleagues [27] demonstrated, in children with severe autism and aggressive behavior, that serum sAPP- α levels were more than twice that of children without autism and up to four times higher than observed in children with mild autism. Bailey [28] measured plasma sAPP- α levels in 25 autistic and age-matched control blood samples using sAPP- α ELISA method and found a significantly increased level of sAPP- α in 60% of the known autistic children, compared to healthy age-matched 2-4 years old children.

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In 2011, Ray [29] investigated 6 mild moderate autistic children, 15 with severe autism and 18 normal children. Their blood samples were analyzed by ELISA method. The results showed that the levels of serum sAPP- α in patients with severe autism were significantly higher, while BDNF, A β 1-40, A β 1-42 and sAPP- β were significantly decreased, as compared with normal. There was no significant difference between the mild-moderate autism children and the normal control group. Following the discovery of multiple secretase activities, sAPP- α was found to mediate the majority of neuronal-enhancing effects and leads to macrocephaly. The study suggests that sAPP- α is expected to be a specific screening indicator for early diagnosis of autism and it still needs large sample and cross-cultural study.

Previous studies show the importance of APP and its cleavage products in the early diagnosis of autism. However, the related study is rare in Chinese autism children. Therefore, our study was designed to explore the possibility of BDNF and sAPP- α as biomarkers for diagnosis of autism in a large group of Chinese autism children with well-matched normal control children. Our studies have concluded that there is a significant increase in plasma sAPP- α expression in early childhood of autism patients, as compared with normal control children, but the expression of plasma BDNF is not significantly different like that of sAPP- α . The significance of this study is particularly important in the context of the effects of the sAPP- α on the nerve nutrition, the abnormal growth of the specific brain regions associated with autism, and the lack of obvious changes in the brain tissue.

Although sAPP- α has not yet been proven to be directly pathogenic for ASD, as a plasma biomarker, it may help delineate a subset of children in which early regional brain overgrowth is necessary and sufficient for the development of autism and may even represent a mechanism by which overgrowth may occur. Indeed it has previously been shown that sAPP- α is able to potentiate nerve growth factor (NGF)/retinoic acid (RA)-induced transdifferentiation of bone marrow-derived adult progenitor cells (MAPCs) into neural progenitor cells and, more specifically, augments their differentiation into a cholinergic-like neuronal phenotype [30]. Interestingly, cholinergic hypertrophy is a common feature of autism [27]. Whether the neuro-

trophic ability of this peptide is present during neurogenesis remains an unanswered key question.

Our finding that plasma sAPP- α level is significantly higher in the patients with severe autism than that in the control group and mild to moderate autism further support our hypothesis that sAPP- α may be the cause of the abnormal synthesis of the central nervous system caused by the abnormal growth of autism. These results suggest that the non-amyloid protein metabolic pathway may exist in severe autism, which results in increased level of sAPP- α , leading to the excessive growth of the synthetic metabolism, and ultimately promote the growth of brain volume in children with autism.

Our study indicate that premature, pathological jaundice, ischemic encephalopathy, birth weight less than 2 kg, pregnancy infection are the impact factors of increased sAPP- α expression. The association between infection and ASD was further supported by the rodent infection model, in which, the offspring of female who experience infection while pregnant have an increased risk for Schizophrenia and autism by causing the innate immune response. The maternal immune response in pregnancy caused by virus infection through IL-6 would disrupt fetal brain development [31]. The results of this study suggest that sAPP- α is much higher in the infection group than those of the control, which supports the view that the perinatal infection promote the occurrence of ASD. Elgen's large sample case control study suggests that low birth weight can increase the risk of ASD by several times [32]. Previous studies have suggested that the brain damage caused in perinatal period is a major factor in the development of autism. The long time effect of infection injury may appear after a period of normal development of the individual [33].

The parental childbearing age greater than 35 years old were introduced into the multiple linear regression models. This is because of aging in parental germ cells, which is susceptible to viral infection, radiation, noise, microwave radiation, environment pollution, and smoking, alcoholism and other damage, resulting in germ cell meiotic abnormal mitosis, fetal malformation rate. At the same time, older mother are prone to complications such as gestational diabetes, pregnancy induced hypertension, and

cardiovascular disease. These factors can lead to the poor development of embryos, such as Embryo damage or pregnancy failure, placental abruption, abortion, premature delivery and so on. Although mild patients have a normal prognosis, severe ones may result in brain damage in early neonatal, death or irreversible brain damage, including low intelligence, cerebral palsy, epilepsy and ataxia, brain dysfunction, autism [34].

Low birth weight, premature birth and low gestational age are the end result of the poor intrauterine fetal growth, and the poor development of nutrition is often associated with brain injury. Parental childbearing age, gestational diabetes, pathological jaundice, pregnancy infection, family history of mental illness and previous ASD history [35, 36] may be risk factors for ASD. The influence factors of sAPP- α and ASD were not the same, but it seemed to overlap. Further research is needed to study whether the risk factors of sAPP- α could be used to establish a disease risk model to predict the early occurrence of ASD in order to improve the prognosis of the disease.

The great difficulty of our understanding the mechanism underlying the cause and development of autism is largely due to the complexity of the genetic basis, the complexity of the childhood normal and abnormal behavior, and a lack of satisfied autism animal model and quantitative markers for diagnosis and prognosis. It is important to improve the early intervention and long-term performance of autism on the base of early diagnosis [37]. Biological markers may help to identify sick children in the perinatal and neonatal period, including the use of cord blood for early screening for autism, like the early screening for other diseases such as congenital hypothyroidism and Down syndrome. In addition, the use of biomarkers associated with autism, combined with other already recognized early behavioral markers, can be used to classify different subtypes of the disease and use specific treatment strategies and therapeutic targets. The facts that only a small amount of biomarkers can be used as a reference and the molecular pathophysiology of autism is only in its early stage greatly hinder the development of more effective therapeutic interventions.

In summary, identifying and characterizing biomarkers such as plasma sAPP- α , combined

with analysis of multiple impact factors will help to create a new ASD diagnostic system using biomarkers and clinical symptoms, and new specific treatment strategies and therapeutic targets based on classification of different subtypes of the disease.

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Disclosure of conflict of interest

None.

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