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Clinical Utility of Salivary Cytokines for Children with Attention Deficit Hyperactivity Disorder

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***Abstract*—**The goal of this study was to examine the possibility of salivary cytokines for the screening of attention deficit hyperactivity disorder (ADHD) in children. We carried out a case-control study, including 19 children with ADHD and 17 healthy children (controls). A multiplex bead array immunoassay was used to conduct a multi-analysis of 27 different salivary cytokines. Six salivary cytokines (interleukin (IL)-1β, IL-8, IL12p70, granulocyte colony-stimulating factor (G-CSF), interferon gamma (IFN-γ), and vascular endothelial growth factor (VEGF)) were significantly associated with the presence of ADHD ($p < 0.05$). An informative salivary cytokine panel was developed using VEGF by logistic regression analysis (odds ratio: 0.251). Receiver operating characteristic analysis revealed that assessment of a panel using VEGF showed "good" capability for discriminating between ADHD patients and controls (area under the curve: 0.778). ADHD has been hypothesized to be associated with reduced cerebral blood flow in the frontal cortex, due to reduced VEGF levels. Our study highlights the possibility of utilizing differential salivary cytokine levels for point-of-care testing (POCT) of biomarkers in children with ADHD.

***Keywords*—**Cytokine, saliva, attention deficit hyperactivity disorder, child, biomarker.

I. Introduction

ADHD is a prevalent neurodevelopmental condition that manifests as sustained attention deficit and/or hyperactivity and impulsive behavior patterns [1]. The 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) is used for the formal diagnosis of ADHD. ADHD impairs quality of life for both children and adults. Pharmacotherapy is the cornerstone for treating ADHD [2]. If estimation of response to chemical treatment by using a biomarker is possible, it will be an effective means of treatment of ADHD.

Inflammatory cytokines associated with ADHD may affect neuronal development directly or via epigenetic pathways [3], [4]. Darwish et al. found significantly higher levels of serum IL-6 in children with ADHD than controls [5]. Further, ADHD patients are reported to have a cerebrospinal fluid cytokine profile intermediate to that of obsessive-compulsive disorder patients and schizophrenia, with somewhat elevated concentrations of tumor necrosis factor alpha (TNF-α) and reduced levels of IFN-γ [6]. However, there have been few studies of the relationship between ADHD and inflammatory cytokines [7]. A salivary cytokine panel to assess different stress responses would be a promising tool for noninvasive monitoring of the stress status in individuals with ADHD. The purpose of this study was to develop a panel of salivary cytokines that manifests the presence of ADHD in children that could eventually improve prognosis by facilitating the diagnosis and management of this disease.

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II. Materials and Methods

A. Participants

The Ethics Committee of Shinshu University School of Medicine (Japan) approved this study. We carried out a case-control study using 19 children with ADHD symptoms (ADHD patients) who met diagnostic criteria based on the ADHD Rating Scale-IV (ADHD-RS) including 16 children with ADHD and 3 inattentive children (mean ± SD: 10.6 ± 2.6 years, Table I). 17 healthy children (mean ± SD: 11.5 ± 3.1 years) who did not meet the ADHD-RS diagnostic criteria were included as controls. Study participation was voluntary, and the study protocol was fully explained to the subjects and their guardians (biological or adoptive parents). Informed consent was obtained from both the children and their guardians.

TABLE I
Summary of the Background of the Subjects

Background	ADHD patients	Controls	p-value
Sample number	19	17	–
Male/Female	12/7	11/6	0.096 [*1]
Age (years)	10.6 ± 2.6	11.5 ± 3.1	0.425 [*2]

[*1] Pearson's chi-square (χ^2) test was conducted.
[*2] Mann-Whitney test was conducted.

B. Saliva Sample Collection

It is possible for chronic periodontitis to influence the concentrations of inflammatory and anti-inflammatory cytokines [8]. It is necessary to exclude any active periodontal disease before procuring a biospecimen. A test paper-strip method (Perioscreen; Sunstar Inc., Osaka, Japan) was used to detect occult blood in saliva [9]. Saliva samples were collected once during the daytime (ADHD patients: 10:00 – 12:35 h, controls: 11:00 – 16:00 h). Subjects did not drink or chew gum in the 30 min before saliva collection. Approximately 150 mL of whole saliva was collected during 1 min [9]. Collected saliva samples were stored at –80 °C and thawed at 4 °C in a refrigerator just prior to analysis.

C. Multiplex Analysis of Salivary Cytokines

We used a multiplex bead immunoassay assay system (Bio-Plex) that has previously been used to examine cytokines

[10], [11]. According to the manufacturer's instructions, 27 different salivary cytokines were analyzed as IL-1 receptor antagonist (IL-1ra), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL12p70, IL-13, IL-15, IL-17A, Eotaxin (CCL11), fibroblast growth factor 2 (FGF-2), G-CSF (CSF3), granulocyte-macrophage colony-stimulating factor (GM-CSF; CSF2), IFN-γ, TNF-α, Interferon-inducible protein 10 (IP-10; CXCL10), monocyte chemotactic protein 1 (MCP-1; CCL2), macrophage inframmatory protein 1α (MIP-1α; CCL3), MIP-1β (CCL4), platelet-derived growth factor-BB (PDGF-BB), regulated on activation, normal T cell expressed and secreted (RANTES), and VEGF [9]. The multiplex bead immunoassay assay system used human cytokine panels (Cat. #M500KCAF0Y, Bio-Plex Pro Human Cytokine Grp I Panel 27-Plex, Bio-Rad Laboratories, Inc., Tokyo, Japan) and the plates were read on a Bio-Plex Array Reader (Bio-Plex 200 System and Bio-Plex Manager Version 6.1, Bio-Rad Laboratories, Inc., Tokyo, Japan) [9].

D.Statistical Analysis and Construction of Prediction Model

Statistical analyzes were performed using a software (IBM SPSS version 25, Advanced Analytics, Inc. Tokyo, Japan). Statistical analysis was not performed if the number of data points missing from one group exceeded half of all data points (> 10 in ADHD patients and > 9 in controls) [12]. Unless otherwise stated, data are expressed as the mean ± standard deviation (SD) [12]. A value of $p < 0.05$ was taken to represent statistical significance [12]. For statistical analyses, the salivary cytokine levels were subjected to log-transformation and standardization; those less than the limit of detection were given a value of half the limit of detection [13], [14].

E. Construction of Prediction Model

Prior to the construction of a prediction model, we compared mean salivary levels of each cytokine between cases and controls by using a multiple linear regression analysis with adjustment for gender and age [12].

TABLE II
COMPARISONS OF LEVELS OF MULTIPLE CYTOKINES BETWEEN ADHD PATIENTS AND CONTROLS BY MULTIPLE LINEAR REGRESSION ANALYSIS

Cytokine	Serum (pg/mL)												p-value[*1]	N of LOD[*2] ADHD/Control
	ADHD patients						Controls							
	Mean±SD			Max-Min		Min	Mean±SD			Max-Min		Min		
IL-1ra	1521.26	±	1152.23	4777.29	-	117.17	2092.83	±	1182.80	4286.05	-	499.44	0.097	0/0
IL-1β	64.57	±	182.51	827.84	-	0.48	84.84	±	120.59	400.29	-	7.67	0.013	0/0
Il-2		—			—			—			—		—	—
IL-4		—			—		0.32	±	0.35	1.20	-	0.02	—	—
IL-5	1.43	±	0.96	3.24	-	0.21	1.32	±	1.46	5.01	-	0.04	—	—
IL-6	13.49	±	37.42	166.57	-	0.13	7.43	±	7.75	27.91	-	0.09	0.126	0/0
IL-7	9.91	±	9.27	40.24	-	0.60	14.35	±	9.61	44.13	-	5.12	0.053	0/0
IL-8	227.43	±	467.46	2136.34	-	9.88	521.90	±	570.18	1996.27	-	75.11	0.002	0/0
IL-9	6.00	±	6.32	24.03	-	0.14	7.36	±	5.45	22.58	-	2.15	0.086	0/0
IL-10	1.77	±	1.76	5.25	-	0.01	1.79	±	1.52	5.58	-	0.24	0.415	0/3
IL-12p70	23.66	±	16.37	56.04	-	5.18	30.32	±	9.49	46.97	-	18.39	0.025	0/0
IL-13	0.45	±	0.50	1.77	-	0.05	1.37	±	3.94	17.09	-	0.06	0.538	0/1
IL-15		—			—			—			—		—	—
IL-17A		—			—			—			—		—	—
Eotaxin	4.18	±	3.58	13.32	-	1.00	4.40	±	4.24	15.41	-	0.63	0.750	0/1
FGF-2		—			—			—			—		—	—
G-CSF	32.88	±	106.85	472.74	-	0.30	32.72	±	46.34	176.27	-	4.24	0.021	0/0
GM-CSF		—			—			—			—		—	—
IFN-γ	16.58	±	24.62	97.72	-	0.12	22.95	±	19.76	84.27	-	2.23	0.031	0/0
TNF-α	13.58	±	19.37	79.09	-	2.46	14.04	±	10.84	39.18	-	4.79	0.283	0/0
IP-10	608.28	±	658.92	2328.26	-	39.69	913.56	±	1015.11	3522.30	-	13.36	0.910	0/0
MCP-1		—			—		32.37	±	31.74	106.15	-	1.52	—	—
MIP-1α	2.60	±	4.13	16.32	-	0.23	1.88	±	1.18	4.92	-	0.68	0.307	0/0
MIP-1β		—			—		12.50	±	16.51	63.51	-	0.44	—	—
PDGF-BB		—			—		4.50	±	5.67	19.94	-	0.01	—	—
RANTES		—			—			—			—		—	—
VEGF	1133.80	±	2020.69	7783.85	-	22.73	3080.37	±	2596.96	8486.07	-	270.78	0.002	0/0

[*1] The multiple linear regression analysis was conducted after adjustment for gender and age.
[*2] The total number of plasma cytokine levels which were less than the limit of detection (LOD).

After adjustment for gender and age, a multiple logistic regression analysis was conducted to examine the association between salivary cytokine levels and the presence of ADHD. For each salivary cytokine, odds ratios (OR) and their 95% confidence intervals (95% CI) were estimated for the presence of ADHD [12].

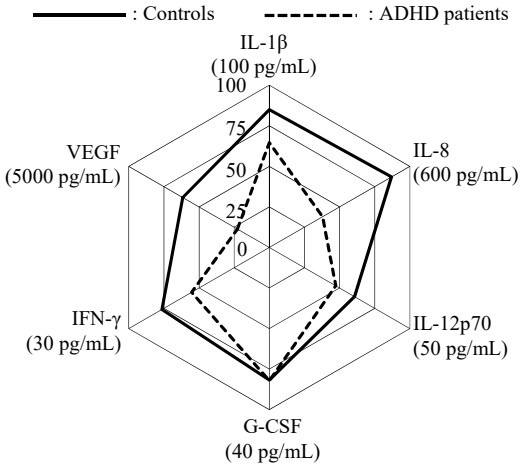

Fig. 1 Comparison of the levels of six salivary cytokines between ADHD patients and controls, significant differences were detected by multiple linear regression analysis

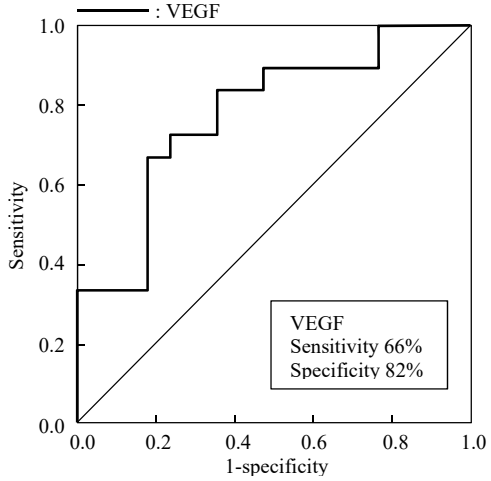

Fig. 2 ROC analysis of the presence of ADHD using the selected salivary cytokine, VEGF, most strongly associated with ADHD

For the construction of a prediction model, candidate salivary cytokines were selected according to the following procedures: To avoid collinearity among variables, the variance inflation factor (VIF) was calculated with multiple regression analysis using physical properties as independent variables and the tactile sensations as dependent variables [15]. A VIF value > 10 indicates multicollinearity among independent variables. We constructed a prediction model for the presence of ADHD by applying a backward elimination method to a logistic regression model under the condition of fixed variables of gender and age. Firstly, we nominated the salivary cytokine that showed the smallest p-value in the multiple logistic regression analysis as the initial candidate from all possible candidate salivary cytokines. Then, another candidate cytokine with the next smallest p-value was identified. These procedures were repeated until candidate cytokines were finalized [12].

III. Results

Table II shows the results of analysis of multiple salivary cytokines in the ADHD patients and controls. In ADHD patients, salivary cytokine levels ranged from 0.01 (IL-10) to 7,783.85 (VEGF) pg/mL. In contrast, the salivary cytokine levels in controls ranged from 0.01 (PDGF-BB) to 8,486.07 (VEGF) pg/mL. A five-digit difference was observed in the absolute values of salivary cytokine levels.

TABLE III
Calculated Outcomes of the Logistic Regression Analysis after Adjustment for Gender and Age

Independent variable	Regression coefficient	p-value	Odds ratio (OR)	95% confidence interval (95% CI)
VEGF	-1.383	0.006	0.251	0.094 - 0.673
Gender	0.556	0.465	1.744	0.392 - 7.752
Age	-0.030	0.606	0.971	0.867 - 1.087

Of the target 27 salivary cytokines, only 16 could be analyzed in ADHD patients and controls, owing to a manifest lack of sensitivity of the Bio-Plex system. The association of these 16 cytokines with presence of ADHD was evaluated by multiple linear regression analysis, after adjusting for potential confounding factors, including gender and age. Six salivary cytokines, namely IL-1β, IL-8, IL12p70, G-CSF, IFN-γ, and VEGF, were significantly associated with the presence of ADHD (Fig. 1, $p < 0.05$).

As shown in Table II, VEGF showed the smallest p-values (< 0.00001) and was selected as the initial candidate for inclusion in the model. Five salivary cytokines were nominated as additional candidates for the model. Finally, the backward elimination method generated a prediction model (logistic model) for the presence of ADHD as ($p < 0.05$):

$$z = -1.450\ \text{VEGF} + 0.494\ \text{gender} - 0.032\ \text{age} \quad (1)$$

We performed receiver operating characteristic (ROC) analysis to closely evaluate the above the prediction model for its ability to detect the presence of ADHD (Fig. 2). The area under the curve (AUC), sensitivity, and specificity at the optimal cutoff point were 0.778, 0.667, and 0.827, respectively (Table III). Thus, the model exhibits "good" capability for to discriminate between ADHD patients and controls.

IV. Discussion

ADHD is a common neurobehavioral condition in children; however, its specific etiology and pathophysiology remain incompletely understood. The present study investigated the role of cytokines and the immune system in the pathogenesis of ADHD, through multiplex analysis of salivary cytokines in children with and without ADHD.

The results support our working hypothesis that the levels of some salivary cytokines vary depending on the presence of ADHD in children. Even after controlling for gender and age, the salivary levels of six cytokines, IL-1β, IL-8, IL12p70, G-CSF, IFN-γ, and VEGF, differed significantly between ADHD patients and controls (Fig. 1). Further, VEGF levels showed promising ability to detect ADHD, with ROC analysis

showing that the VEGF model had "good" capability to discriminate between ADHD patients and controls (Table III and Fig. 2).

In our research, logistic regression analysis revealed that the cytokine, VEGF, is potentially associated with the presence of ADHD. VEGF is produced by cells that stimulate the formation of blood vessels, and is an important angiogenic cytokine [16]. Equation (1) indicates that ADHD is negatively correlated with VEGF levels. This result is consistent with the findings of a previous study, which indicated that cerebral blood flow in the frontal cortex could be reduced in ADHD patients due to the lowered levels of VEGF [17].

V. Conclusion

In our study, we found that the levels of several salivary cytokines varied significantly between children with and without ADHD, suggesting a possibility of using the differential expression of salivary cytokines as potential biomarkers for detecting the presence of ADHD. Our results should be validated in other populations and supported by future studies to examine the temporal changes in salivary cytokine levels with ADHD progression and prognosis. These results support the notion that cytokines may play a role in the selective cognitive and behavioral symptoms of ADHD. Further these data have the potential to contribute to the realization of POCT systems for clinical implementation [18]. Further investigations are required to elucidate the correlations of cytokines at different sites, such as the plasma, saliva, and brain, with ADHD.

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