Research Article

Ihsan Cetin*, Hamdullah Bulut and Şeref Şimsek

Elevated serum ubiquitin-proteasome pathway related molecule levels in attention deficit hyperactivity disorder

Dikkat eksikliği hiperaktivite bozukluğunda artmış serum ubikutin-proteazom yolu ile ilişkili molekül düzeyleri

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Abstract

Introduction: We aimed to determine the serum levels of transactive response of DNA-binding protein 43 (TDP-43) and ubiquitin C-terminal hydrolase-L1 (UCH-L1), which are ubiquitin-proteasome pathway related molecules and have not been investigated so far, in children with attention-deficit/hyperactivity disorder (ADHD).

Methods: The study group was composed of thirty children aged between 6 and 10. They were diagnosed with ADHD according to DSM-IV criteria. They were the subjects who applied to Dicle University, Faculty of Medicine, and Department of Child Psychiatry in Diyarbakır, Turkey. Children with ADHD were assessed via Turgay DSM-IV Based Child and Adolescent Behavior Disorders Screening and Rating Scale and Stroop test. Serum TDP-43 and UCH-L1 levels were analysed with enzyme-linked immunosorbent assay.

Results: The TDP-43 and UCH-L1 serum levels of children with ADHD were found to be statistically significantly higher than those of controls. On the other hand, we found that serum levels of TDP-43 correlated with interference effect and hyperactivity–impulsivity in children with ADHD.

Conclusion: Imbalances in serum UCH-L1 and TDP-43 levels, and the correlation of TDP-43 levels with clinical parameters in children with ADHD may suggest that ubiquitin-proteasome pathway alterations are associated with ADHD. Deterioration of this pathway may cause intracellular TDP-43 aggregation.

Keywords: ADHD; TDP-43; UCH-L1; Neurodegeneration; Ubiquitination.

*Corresponding author: Ihsan Cetin, Batman University, School of Health, Batman – Department of Nutrition and Dietetics, Batman, Central 72060, Turkey, e-mail: ihsancetinilim@gmail.com; ihsan.cetin@batman.edu.tr

Hamdullah Bulut: Batman Universitesi – Institute of Science, Batman, Turkey

Şeref Şimsek: Dicle University, School of Medicine – Department of Child and Adolescent Psychiatry, Diyarbakır, Turkey

Önemli:

Giriş: Dikkat eksikliği hiperaktivite bozukluğu (DEHB) olan çocuklarda ubikutin-proteasom süreciyle ilişkili olan ve daha önce incelenmemiş transaktif yanıt DNA bağlayıcı protein 43 (TDP-43) ve ubikutin C-terminal hidrolaz-L1 (UCH-L1) molekülerinin serum seviyelerini belirlemeyi amaçladık.

Yöntem: Çalışma grubu 6 ile 10 yaş arasındaki otuz çocuktan oluşturuldu. Çocuklara DSM-IV kriterlerine göre DEHB tanısı konuldu. Çocuklar Diyarbakır Dicle Üniversitesi Tip Fakültesi, Çocuk Psikiyatri Bölüümü’ne başvuran kişilerdi. DEHB’i olan çocuklar, Turgay DSM-IV dayalı çocuk ve her iki otuz tarama ve değerlendirmeye ölçüprü ve stroop testi ile değerlendirildi. Serum TDP-43 ve UCH-L1 düzeyleri enzim bağlı immunsorbent ölçüm ile analiz edildi.

Bulgular: DEHB olan çocuklarında TDP-43 ve UCH-L1 serum seviyelerinin kontrol grubuna göre istatistiksel olarak anlamlı derecede yüksek olduğu tespit edildi. Bununla birlikte, DEHB olan çocuklarda TDP-43 serum düzeylerinin interferans skoru ve hiperaktivite-diürtüsel olgular ile korelasyon gösterdiği bulunmuştur.

Sonuç: DEHB olan çocuklarda serum UCH-L1 ve TDP-43’in yüksek düzeyleri ve TDP-43 seviyelerinin klinik...
Introduction

Attention deficit hyperactivity disorder (ADHD) afflicted individuals exhibit inability to inhibit behavioural, antisocial, depressive symptoms of the disorder [1]. The experimental and clinical studies suggest that pathophysiology and neurobehavioural deficiencies of ADHD are related to microstructural abnormalities [2], glial-derived neurotrophic factor [3], apoptotic neurodegeneration [4], microglial activation [5] and neurotrophin reflecting glial integrity (S100B) [6]. Moreover, it is suggested that ADHDs are highly associated with genes recognized as prominent for neurological functions such as synaptic transmission, learning and behavior [7]. Accordingly, impairments in learning, delayed executive and memory functions are reported in ADHD [8].

Ubiquitin-proteasome pathway (UPP) plays a crucial role in forming synaptic connections during the development of nervous system and it is also identified as a molecular mechanism managing numerous neuron functions [9]. Ubiquitin C-terminal hydrolase-L1 (UCH-L1) is specifically expressed in neurons and plays an important role in the process of removal of misfolded proteins via UPP [10]. Aggregates of Transactive Response DNA-Binding Protein (TDP-43) appear to be degraded through both UPP and autophagy [11]. TDP-43 is also appointed as an ingredient of ubiquitin-positive inclusion in brains with neurodegenerative diseases [12]. In some specific TDP-43 transgenic models, cognitive impairments, progressive motor behavioral deficits, memory and learning deficits in particular are also detected [13–15]. Furthermore, it is demonstrated that overexpression of UCH-L1 improves memory deficits and inhibits neuritic plaque formation in transgenic mice model of Alzheimer’s disease [16]. Overexpression of UCH-L1 recovering memory and healing learning impairments in mice with Alzheimer’s disease by restoring long-term potentiation in the hippocampus [17] and reducing UCH-L1 protein levels are also found in sporadic Alzheimer’s disease brains [18].

Cognitive studies found deficits in both adults and children with ADHD with impairments on executive functions, particularly in those with sustained attention and measuring response inhibition [19]. Moreover, it was determined that a pattern of marked regional brain cell degeneration was related with deficits expressed-inattention, hyperactivity and dishabituation in the adult animals [4]. This could be in connection with the impairments and/or roles of neural substrates in behavioural inhibition, working memory, decision making and emotional symptoms of adult patients with ADHD [20, 21].

Better understanding the biochemistry and the genetics of ADHD will contribute to the knowledge necessary for developing other cures for ADHD [15]. Lately, neural models of ADHD have switched the focus from the investigation of local brain abnormalities to distributed connectivity in networks [22, 23]. Considering these aspects, we hypothesized that UPP might be affected by ADHD in children and the system related to molecular levels could be increased or decreased in ADHD. On the other hand, the TDP-43 and UCH-L1 were identified as two important molecules associated with UPP [9–18]. We, therefore, proposed that the quantification of extracellular TDP-43 and UCH-L1 in serum could offer an opportunity for the development of a molecular biomarker for ADHD. As a result, we set out to investigate serum levels of TDP-43 and UCH-L1 that have not been searched in children suffering from ADHD up until now.

Materials and methods

Study groups

The study group included 30 children diagnosed with ADHD aged 6 to 10 with regard to DSM-IV criteria. These children applied to Department of Child Psychiatry in Faculty of Medicine of Dicle University, Diyarbakir, Turkey. Thirty subjects, who did not have any psychiatric disorders and medical history, and matching in age and gender, were chosen as healthy control group. The parents of children gave written consent for their children’s participation in this voluntary investigation. Children with ADHD, having comorbidity with behavioural and oppositional disorders, were also included in the study. Ethics Committee of Dicle University affirmed the study protocol (Date/Decision Number: 27.02.2015/167).

Tests, scales and assessments

Kiddie-Sads-Present and Lifetime Version (K-SADS-PL) was conducted via conversing to children’s parents;
consequently, summary ratings including all information sources were procured. Gökler et al. [26] adapted this test for children and adolescents living in Turkey. K-SADS-PL is a semi-structured diagnostic interview envisaged to evaluate present and previous episodes of psychopathology in children and adolescents with respect to DSM-IIIR and DSM-IV criteria [25].

Turgay DSM-IV Based Child and Adolescent Behavior Disorders Screening and Rating Scale (T-DSM-IV-S) was individually administered. This scale was developed by Turgay [26] according to DSM-IV criteria, consisting of 41 items. Of these items, nine were administered individually for questioning attention deficiency, six for hyperactivity, three for impulsivity, eight for oppositional defiant disorder, and 15 for behavior disorders.

Stroop test, reflecting the frontal area of operations, was applied to children with ADHD but for the control group. In Stroop test, resistance to interference and processing speed were evaluated by making the participant pay attention selectively to the task-relevant stimulus and to respond this stimulus and inhibit a competing automatic response [27]. The timing, number of corrections and mistakes were evaluated. Children diagnosed with psychiatric and neurological disorders such as mental retardation, history of seizures, a history of encephalitis, pervasive developmental disorder, chronic systemic disease and pervasive developmental disorder intelligence scores of lower than approximately 70 were left out of the investigation [28].

Biological measurements/assays

Three different tubes without anticoagulant were used to draw blood samples via venipuncture technique. This technique was administered not only for control but also for patient groups after an overnight (≥ 12 h) fast. These blood samples were let to clot for 30 min. Samples were centrifuged at 4000 rpm for 10 min. Hence, it was possible for all large particles and blood cells in blood specimens to be precipitated. For further investigation, clear yellow serum samples were picked up. Not only lipemic but hemolyzed samples were removed as well. The aliquots of serum samples were stored at –70°C until TDP-43 and UCH-L1 concentration measurements.

Serum levels of TDP-43 and UCH-L1 were determined with ELISA method (YEHUA Biological Technology, with catalogue numbers YHB2918Hu and YHB3139Hu, respectively). Briefly, 50 μL standards were added in standard solution wells, 40 μL serum samples and 10 μL TDP-43 or UCH-L1 antibodies were added in specimen wells. Afterwards, 50 μL streptavidin-HRP was added to wells except for blank well. Then the plate was covered by a transparent membrane. In order to mix, the plate was shaked kindly and incubated. Then the plate was carefully washed for five times. Fifty microlitre each of chromogenic reagent A and B was added. And later the plate was incubated. The optical density (OD) of wells was measured at 450 nm after stop solution was added. Then, the linear regression equation for the standard curve was calculated according to OD values of standard concentrations. TDP-43 and UCH-L1 concentrations of samples were established. Intra-Assay coefficient of variation (CV) was found to be <10% and inter-Assay CV <12% for both parameters. Assay ranges were 20 ng/L–6000 ng/L for TDP-43 and 1 ng/mL–38 ng/mL for UCH-L1.

Statistical analyses

Statistical analyses were carried out by making use of statistics program, SPSS Statistics software 22.0. The data normality was evaluated by the Q-Q graphs and Shapiro Wilk normality test. Group mean age differences were examined by means of unpaired t-test. By means of exact method of the χ²-test, gender comparison was conducted. Mann-Whitney U-test was used for comparison of TDP-43 and UCH-L1 levels between groups. Correlation analyses

Table 1: The basic characteristics, Stroop test and T-DSM-IV-S scores in study groups.

| Parameters                  | Control group (n=30) | ADHD group (n=30) | p-Value |
|-----------------------------|----------------------|-------------------|---------|
| Gender (F/M)                | 15/15                | 14/16             | >0.05   |
| Age, years                  | 8.5 ± 1.4            | 8.9 ± 2.2         | >0.05   |
| Interference effect         | –                    | 21.4 ± 6.8        | –       |
| Attention                   | –                    | 17.7 ± 4.3⁵       | 16.4 ± 5.0¹ | –       |
| Hyperactivity–impulsivity   | –                    | 12.8 ± 8.6⁶       | 13.5 ± 5.5¹ | –       |
| Opposition defiance         | –                    | 9.8 ± 7.7⁷        | 10.3 ± 7.1¹ | –       |

T-DSM-IV-S: Turgay DSM-IV Based Child and Adolescent Behavior Disorders Screening and Rating Scale. F, female; M, male; ⁵according to parents; ¹according to teachers.
between clinical and molecular parameters were studied with the help of Spearman's test.

**Results**

The control group of our study included 30 subjects (15 males and 15 females) and 30 children with ADHD (16 males and 14 females). The mean age of the controls was 8.5 ± 1.4, while it was 8.9 ± 2.2 with the ADHD children. Controls and children with ADHD did not show any statistically significant differences in terms of age and gender (p > 0.05; Table 1). Stroop test and Behavior Disorders Screening and Rating Scale results of children with ADHD are shown in Table 1. Interference effects of ADHD, obtained by applying the Stroop test, were 21.4 ± 6.8. The mean attention scores of children with ADHD reported by parents and teachers were 17.7 ± 4.3 points and 16.4 ± 5.0 points, respectively. The mean hyperactivity–impulsivity scores of children with ADHD reported by parents and teachers were 12.8 ± 8.6 points and 13.5 ± 5.5 points, respectively. The mean opposition defiance scores of children with ADHD reported by parents and teachers were 9.8 ± 7.7 points and 10.3 ± 7.1 points, respectively. Categorical changables were stated as number; and constant variables were expressed as mean ± SD or median (25th–75th percentile) (Table 1).

The TDP-43 levels [840.23 (597.5–3432.3) ng/L] of children with ADHD were found to be significantly (p = 0.022) higher than those of controls [770.8 (114.6–1293.2) ng/L] (Figure 1). Similarly, the UCH-L1 levels [7.46 (3.97–30.2) pg/mL] of children with ADHD were found to be significantly (p < 0.001) higher than those of controls [4.97 (2.90–12.0) ng/mL] (Figure 2).

TDP-43 and UCH-L1 concentrations did not show any statistically significant (p > 0.05) differences with respect to age and gender in the study groups.

It was established that TDP-43 serum distribution levels in healthy children and children with ADHD were min = 114.6, max = 1485.3 and min = 597.5, max = 4644.6, respectively. UCH-L1 serum distribution levels in healthy

**Figure 1:** Tukey box plots and scatter plots illustrating the results of the categorical comparison between control and ADHD groups in terms of TDP-43 concentrations (p = 0.002).

**Figure 2:** Tukey box plots and scatter plots illustrating the results of the categorical comparison between control and ADHD groups in terms of UCH-L1 concentration (p < 0.001).
children and children with ADHD were $\text{min} = 2.90$, $\text{max} = 12.18$ and $\text{min} = 3.97$, $\text{max} = 30.15$, respectively (Table 2).

As a result of correlation analysis between ubiquitin-proteasome pathway related molecule levels and clinical characteristics, we found that serum TDP-43 levels were significantly positively correlated with interference effect in children with ADHD ($p < 0.05$; Figure 3). Similarly, TDP-43 levels were significantly positively correlated with hyperactivity–impulsivity in children with ADHD ($p < 0.05$; Table 3). Additionally, hyperactivity–impulsivity was positively correlated with opposition defiance in children with ADHD ($p < 0.05$; Table 3).

### Discussion

In this study, it was revealed that serum levels of UCH-L1 and TDP-43 levels were found to be higher in children with ADHD compared to healthy children.

While TDP-43 and UCH-L1 are typically regarded as intracellular proteins, they are, however, normally found in extracellular biological fluids, embracing both human plasma and cerebrospinal fluid (CSF) [29–31]. Due to the direct contact of CSF with the brain interstitial fluid, this great likely supplies a more accurate assessment of TDP-43 and UCH-L1 metabolism than peripheral blood [32]. Nevertheless, as is CSF released from the subarachnoid granulations to the venous circulation, outputs transferred into the CSF from the brain may pass into blood [33].

Considering these aspects, it may be suggested that TDP-43 and UCH-L1 are discharged into the extracellular space and can be detected in serums of children with ADHD and healthy children.

Perhaps, another most important finding of our study was that the demonstration of the TDP-43 levels was correlated with interference effect and hyperactivity–impulsivity in children with ADHD.

### Table 2: Values and distributions of TDP-43 and UCH-L1 in study groups.

| Parameters                  | Control group (n=30)   | ADHD group (n=30)     | p-Value |
|-----------------------------|------------------------|-----------------------|---------|
| TDP-43, ng/L                | 836.3 (685.8–1059.6)   | 868.8 (702.6–2369.9)  | 0.022   |
| UCH-L1, ng/mL               | 4.97 (3.96–7.92)       | 7.46 (5.20–17.45)     | 0.001   |
| TDP-43, ng/L (min-max)      | 114.6–1485.3           | (597.5–4644.6)        | –       |
| UCH-L1, ng/L (min-max)      | 2.90–12.2              | (3.97–30.15)          | –       |

Data are expressed as median (25th–75th percentile) for continuous variables.

![Figure 3: Scatter dot plot illustrating the correlation between TDP-43 (ng/L) and interference effect in children with ADHD ($p: 0.577; p: 0.039$).](image)

### Table 3: Correlation between TDP-43, UCH-L1 and clinical characteristics in children with ADHD.

|                      | TDP-43 | UCH-L1 | Hyperactivity–impulsivity |
|----------------------|--------|--------|---------------------------|
| Interference effect  | 0.577a | 0.484  | 0.520                      |
| Attention            | −0.013 | −0.315 | 0.203                      |
| Hyperactivity–impulsivity | 0.510a| 0.144  | 1                          |
| Opposition defiance  | 0.400  | 0.091  | 0.636b                    |

*p = Spearman correlation coefficient. *Level of statistical significance is smaller than 0.01. Bold values indicate the presence of a statistically significant correlation between parameters.
Previous studies, which were performed on TDP-43 transgenic mice, revealed behavioral deficits, motor impairments, cognitive deficits including memory/learning defects and reduced social interaction. On the other hand, it was shown that higher prevalence of learning disability was determined in children with ADHD than those of the general population [34]. It was demonstrated that the rate of ‘hyperactivity’ in adults increases prominently with rising levels of learning defect [35]. Previous studies also found widespread impairments including interference control, deficits expressed-inattention, hyperactivity, and behavioural inhibition in patients with ADHD [3, 18, 35, 36].

Considering the previous studies [13–15, 34, 35], we contemplate that TDP-43 transgenic mice and individuals affected by ADHD have similar symptoms. Therefore, we suggest that correlations between TDP-43 levels and clinical parameters in children with ADHD may be evidence of the relationship of TDP-43 proteinopathy in children with ADHD.

It was found that UCH-L1 was involved in the processing of degradation of misfolded proteins via the proteasomal pathway [10]. Many studies show that UCH-L1 is to be linked with neurodegenerative disorders in animals and humans. In addition, recent evidences have supported the presence of UCH-L1 in CSF and peripheral body fluids in animal and human in the cases of subarachnoid hemorrhage, and ischemic and traumatic brain injury [10, 16–18].

Similar to previous studies, conducted on different patient groups [29, 31, 37–39], our results showed that the serum TDP-43 and UCH-L1 levels were different and more widely dispersed in children with ADHD than those of healthy children. Nevertheless, we did not find any significant correlations of UCH-L1 with TDP-43 and clinical parameters in children with ADHD. The absence of the correlation may be due to the small number of patients. Therefore, future studies, to be performed on a greater number of participants, should include pathological evaluations of UCH-L1 and TDP-43 with clinical parameters in children with ADHD.

After all, this study has some limitations. Firstly, the small number of subjects may have limited to generalization of our findings. Second, we collected only serum samples, but CSF samples were absent. There is very little information on serum TDP-43 and UCH-L1 levels in peripheral body fluids, so more investigation is needed to approve and improve these studies as it is not entirely clear how serum levels of TDP-43 and UCH-L1 increase in children with ADHD.

In conclusion, we found that children with ADHD had higher serum levels of UCH-L1 and TDP-43 compared to healthy controls. We also found positive correlation between TDP-43 levels with interference effect and hyperactivity–impulsivity in children with ADHD. Our preliminary study may provide evidence for UPP related proteinopathies and the intracellular TDP-43 aggregation in children with ADHD. We consider that it will be of significant help to specify whether or not the TDP-43 and UCH-L1 can be used for the determination of ADHD in children. And also we want to learn whether their increased levels have any phenotypic effect. However, the lack of knowledge about CSF levels of TDP-43 and UCH-L1 is still present; so, the subject deserves further investigation.

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Conflict of interest: There are no conflicts of interest.

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