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Biochemical risk factors associated with refractory epilepsy: alpha synuclein and adenosine deaminase

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ABSTRACT

Background: Epilepsy is a common chronic neurological disorder affecting all age groups. A significant portion of children with epilepsy develop drug-resistant seizures. These children are at risk of cognitive and behavioral comorbidities and death. Some clinical features provide important information about the prognosis of epilepsy. However, currently, there is no objective biochemical indicator associated with refractory epilepsy. This study aimed to determine whether serum alpha-synuclein (αS), p-glycoprotein (P-gp), and adenosine deaminase activity (ADA) were biochemical risk factors for refractory epilepsy.

Methods: The cross-sectional study included patients diagnosed with refractory epilepsy (n=32), non-refractory epilepsy (n=35) and 20 healthy children under the age of 18 who applied to the Pediatric Neurology outpatient clinic. In the serum samples, αS and P-gp were analyzed by ELISA and ADA activity was analyzed by spectrophotometric method. ROC analysis was applied and the area under the curve (AUC) was calculated to define cut-off values in distinguishing refractory epilepsy patients from non-refractory epilepsy patients. Binary logistic regression analysis was performed to define risk factors associated with resistance in patients with epilepsy.

Results: Children with epilepsy whose ADA activity and αS values were higher than the determined cut-off values had 10-fold and 5.3-fold increased risk of refractory epilepsy, respectively.

Conclusions: αS and ADA activity can be used as biochemical risk factors for refractory epilepsy. However, these results need to be confirmed by prospective studies with many patients.

Keywords: adenosine deaminase, alpha-synuclein, p-glycoprotein, refractory epilepsy, risk factors

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INTRODUCTION

Epilepsy is a neurological disease defined by abnormal neurophysiological activity accompanied by varying degrees of loss of sensation or consciousness, leading to epileptic seizures or abnormal behavior. Additionally, its prevalence in children is reported to be 0.5-1% [1]. Despite advances in treatment based on various molecular targets, more than one-third of epilepsy patients still show resistance to anti-epileptic drugs (AEDs) [2]. The underlying etiology of epilepsy, seizure type, number of seizures before starting treatment, and age of the first seizure are among the factors that determine the prognosis of epilepsy [3]. Epilepsy is defined as “two or more unprovoked (or reflex) seizures more than 24 hours apart; "one unprovoked (or reflex) seizure and at least a 60% risk of recurrence within the next 10 years after two unprovoked seizures, or a diagnosis of epilepsy syndrome" is defined as the patient experiencing at least one of these three conditions. The diagnosis of epilepsy is based primarily on the patient's medical history and electrophysiological techniques such as electroencephalogram (EEG) or tissue imaging techniques such as magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT) [4]. The pathophysiological cause of epileptic seizures is the abnormal discharge of neurons seen in EEG [5]. There are no objective biochemical indicators that will provide a practical approach to the follow-up of these patients [6].

In clinical practice, the doctor is almost always faced with an EEG, interictal epileptiform discharge, obtained during the interictal state. EEG monitoring may not necessarily show any specific symptoms attributable to the disease. Despite an underlying epileptic disorder, the EEG may be completely normal, which often leads

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to difficulties in diagnosing the disease. Therefore, biochemical indicators could help shorten the period of diagnostic uncertainty and consequently decrease the risk of seizures [7]. Alpha-synuclein (αS), one of the possible indicators, can form ring-shaped protofibrils that can be incorporated into membranes and form pores and non-specific ion channels, affecting membrane permeability, and leading to cell dysfunction. Permeability glycoprotein (P-gp), another possible indicator, is widely found in the capillary endothelial cells of the blood-brain barrier (BBB) as well as intestinal epithelium, and proximal tubule cells of the kidney. It has been emphasized that P-gp overexpression in the BBB may lead to drug resistance by severely restricting drug penetration into the brain [8]. Finally, adenosine deaminase (ADA) is an enzyme that catalyzes the irreversible deamination of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively and has an important function in regulating adenosine concentration [9]. It is emphasized that adenosine may be an important endogenous neuroprotectant [10].

Some clinical features provide important information about the prognosis of epilepsy. Additionally, biochemical risk factors may make positive contributions to the follow-up and management of treatment. Therefore, this study aimed to determine whether serum αS, P-gp, and ADA activity were biochemical risk factors for refractory epilepsy.

METHODS

Study design and participants

The cross-sectional study took place at the Erciyes University Child Neurology outpatient clinic between April and September 2023, and the study was approved by the Erciyes University Clinical Research Ethics Committee (dated 14.09.2022 and decision number 598/2022). Written informed consent was obtained by informing the patients or their parents and the study was conducted by the principles outlined in the Helsinki Declaration.

The research included patients under the age of 18. a-Patients with non-refractory epilepsy (n=35). b-Patients diagnosed with refractory epilepsy (n=32). c-Healthy control: children applied to the social pediatrics outpatient clinic and did not have any acute or chronic systemic disease and had no history of convulsions or head trauma (n=20).

Exclusion criteria for the study for refractory epilepsy: using drugs with p-glycoprotein inhibitor effect, those with serious drug reactions, presence of pseudo-seizure, liver and kidney failure, and malignant and metabolic disease. Exclusion criteria for the study for non-refractory epilepsy: patients with epilepsy who are in clinical remission with gradual discontinuation of treatment, presence of liver and kidney failure, malignant disease, metabolic disease and for the control group: those with a history of convulsions or head trauma, those using medication.

According to the International Association Against Epilepsy (ILAE), failure to achieve seizure freedom despite two different antiepileptic drugs used alone or in combination at the highest tolerated dose is considered refractory epilepsy. Seizure freedom used in this definition refers to the absence of any seizures in the last 12 months or the absence of seizures three times the longest seizure-free period experienced by the patient in the last 12 months. Non-refractory epilepsy was defined as the patients who have not had a seizure in at least 6 months of follow-up after the diagnosis of epilepsy and the initiation of monotherapy [4]. The patient's age, gender, seizure type, seizure frequency, seizure onset age, treatment history, and treatments were recorded.

Sample collection and laboratory analysis

Blood samples were taken into non-anticoagulant tubes and αS and P-gp levels in separated serum samples were analyzed by ELISA (USNC and SunRed, China respectively). ADA activity was determined spectrophotometrically by the Guisti method [11]. Ammonia released during the conversion of adenosine to inosine under ADA catalysis reacts with phenol and hypochlorite reagents in an alkaline environment to form the blue-colored indophenol compound. ADA activity was calculated by measuring the absorbance of the resulting color at 628 nm and comparing it with standards.

Statistical analysis

The suitability of the data for normal distribution was evaluated with the histogram and the Shapiro-Wilk test. One-way analysis of variance (ANOVA) and the Kruskall-Wallis test were used to compare study groups with and without normal distribution, respectively. Tukey and Dunn-Bonferroni tests were used for multiple comparisons. Values are summarized using the frequencies and percentages, the mean and the standard deviation or median and interquartile range statistics. The Chi-square test was used to compare categorical variables.

Receiver Operating Characteristic (ROC) analysis was applied to define optimal cut-off values in distinguishing refractory epilepsy patients and the area under the curve (AUC) was calculated. Binary logistic regression analysis was applied to determine factors associated with refractory epilepsy. The clinical features were included in the model 1. Biochemical risk factors' potential association with refractory epilepsy was added at a

stringency level of p< 0.05 (Model 2). The model suitability was examined with the Nagelkerke R square and the Hosmer-Lemeshow test. Odds ratios were calculated. The significance level was received as p <0.05. "IBM SPSS Statistics 22" statistical package program was operated to interpret the data.

RESULTS

The mean age of patients in the refractory and non-refractory epilepsy groups were 8.53 ± 4.37 years and 11.00 ± 4.02 years, respectively. The mean age of the control group was 8.95 ± 4.24 years. The gender distribution of the patients (60% boy-40% girl in the non-refractory epilepsy group, 50% boy-50% girl in the refractory epilepsy group) and controls (50% boy-50% girl) was similar. No difference was detected between the groups in terms of age and gender (p>0.05).

The dispersion of patients diagnosed with epilepsy according to their clinical characteristics is presented in Table 1. Seizures started in infancy in 63% of the children with refractory epilepsy and seizures were frequent (daily/weekly) in 56% of these children. Additionally, seizures were mostly generalized but rarely combined-onset seizures were observed. Genetic and structural factors were responsible for the etiology in 66% of the children with refractory epilepsy. A significant number of children with refractory epilepsy have mental retardation, delay in psychomotor development and epileptic syndromes.

αS levels showed significant differences among the groups. αS levels were found to be significantly higher in children with refractory epilepsy than in healthy children (p<0.001), and children with non-refractory epilepsy (p=0.009) (Table 2).

ADA activity was higher in the refractory epilepsy group than in non-refractory epilepsy patients (p=0.008). Again, ADA activity was detected to be significantly higher in the refractory epilepsy group compared to the controls (p<0.001) (Table 2).

To distinguish refractory and non-refractory epilepsy patients, the optimal cut-off values of ADA activity, αS and P-gp levels were determined by ROC analysis and AUC was calculated. The cut-off value for ADA was 20.24, AUC=0.681 (95% Confidence interval (CI) 0.556-0.789), p=0.006; for αS was 0.141, AUC 0.722 (95% CI 0.599-0.824), p<0.001 and finally, the cut-off value for p-gp was determined as 5.07, AUC 0.633 (95% CI 0.506-0.747), p=0.05. A comparison of AUC values in distinguishing resistant and non-refractory epilepsy patients at optimal cut-off values of ADA activity and αS was shown in Figure 1. Accordingly, αS had the highest AUC value.

Table 1. Dispersion of patients with epilepsy according to their clinical characteristics

Variables	Non-refractory epilepsy	Refractory epilepsy
Age at Seizure Onset		
Infant (0-2 years old)	9 (26%)	20 (63%)
Children (3-11)	21 (60%)	11 (34%)
Adolescent (12-18)	5 (14%)	1 (3%)
Epilepsy Type		
Focal onset	5 (14%)	-
Generalized onset	30 (86%)	28 (88%)
Combined focal and generalized onset	-	4 (12%)
Seizure frequency		
Daily/Weekly	-	18 (56%)
Monthly/Annual	35 (100%)	14 (44%)
Etiology		
Infection	2	-
Intracranial hemorrhage	2	1
Genetic disorders	-	9
Hypoxic-ischemic encephalopathy	2	7
CNS malformation	-	2
Microcephaly	-	-
Metabolic	2	2
Other	27 (77%)	11 (34%)
Epileptic Syndromes		
West syndrome	-	3
Lennox Gastaunt syndrome	-	1
Dravet syndrome	-	1
Rett syndrome	-	-
Mental Retardation	4 (12%)	23 (72%)
Psychomotor developmental disorder	6 (17%)	24 (75%)
Number of Antiepileptic Drugs Used		
1	35 (100%)	-
2	-	12 (37.5%)
3	-	12 (37.5%)
>3	-	8 (25%)

Data were presented as n (%). Non-refractory epilepsy (n=35). Refractory epilepsy (n=32).

ADA activity had a sensitivity of 93% and a negative predictive value of 86% in identifying patients with refractory epilepsy. The highest specificity value (85%) was reached when the cut-off for αS was taken as 0.161.

According to binary logistic regression analysis, although older age at first seizure reduced the risk of refractory, epilepsy etiology resulting from a structural, genetic or metabolic cause increased the risk by 5.3-fold. ADA activity and αS levels were determined to be independently associated with refractory epilepsy. Children with epilepsy whose ADA activity and αS values were higher than the determined cut-off values had 10-fold and 5.3-fold increased risk of refractory epilepsy, respectively (Table 3).

Table 2. Comparison of αS, P-gp levels and ADA activity of patient and control groups

Variables	Control (n=20)	Non-refractory epilepsy (n=35)	Refractory epilepsy (n=32)	p value
αS (ng/mL)	0.128 (0.123-0.130[a]	0.128 (0.120-0.151[a]	0.157 (0.133-0.170[b]	<0.001
P-gp (ng/mL)	4.10 (3.91-4.29)	4.17 (3.66-5.07)	5.74 (3.84-8.03)	0.067
ADA (IU/L)	21.46 ± 4.07[a]	24.73 ± 7.57[b]	30.46 ± 8.95[c]	<0.001

Values were expressed as median (1st – 3rd quartiles) or mean ± standard deviation. Significant p values were shown in bold. Different superscripts in the same row indicate a statistically significant difference between groups. Abbreviations: αS- alpha-synuclein; P-gp- p-glycoprotein; ADA- adenosine deaminase.

Table 3. Binary logistic regression

Model	Variables	Beta	Odds ratio	95% Confidence Interval		p value	Nagelkerke R squared
				lower	Upper		
1	Age at the first seizure	-0.270	0.763	0.639	0.911	0.003	41.3%
	Seizure etiology	1.671	5.318	1.607	17.602	0.006	
	Constant	0.354	1.425			0.497	
2	Age at the first seizure	-0.36	0.790	0.644	0.969	0.023	58.5%
	Seizure etiology	1.779	5.923	1.435	24.442	0.014	
	ADA (cut-off 20.24)	2.165	10.159	1.438	71.756	0.020	
	αS (cut-off 0.161)	1.447	5.399	1.384	21.053	0.015	
	Constant	-2.525	0.80			0.032	

Dependent variable: Patient groups: non-refractory epilepsy (0); refractory epilepsy (1). Model 1: Independent variable; Age at the first seizure, seizure etiology (structural conditions such as infection, intracranial hemorrhage, hypoxic-ischemic encephalopathy, CNS malformation; genetic, and metabolic (0); others (1) Model 1: Nagelkerke R square = 41.3%, Hosmer-Lemeshow p=0.625. Model 2: Independent variables; ADA (cut-off 20.24), αS (cut-off 0.161) in addition to age at the first seizure, seizure etiology. Model 2: Nagelkerke R square = 58.5%, Hosmer-Lemeshow p=0.464.

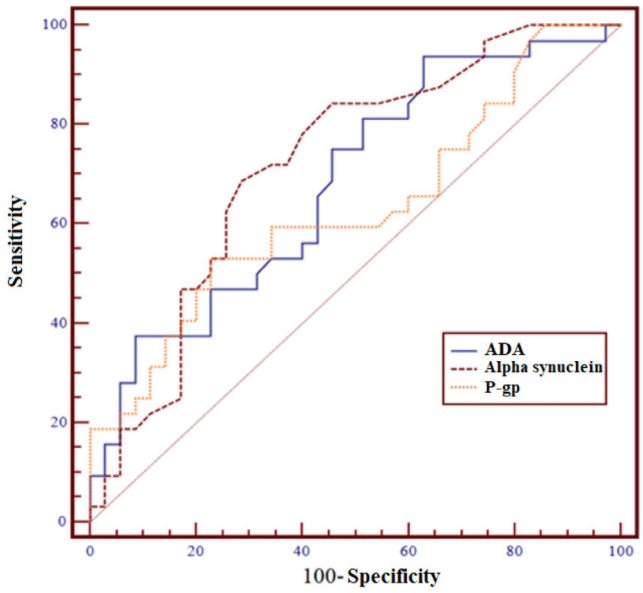


Figure 1. Comparison of ROC curves of adenosine deaminase (ADA), p-glycoprotein (P-gp), and alpha synuclein.

DISCUSSION

Risk factors associated with treatment resistance are based more on the patient's clinical findings and imaging methods. A reliable indicator would be of great practical importance in monitoring patients' response to antiepileptic drugs or assessing the risk of refractory epilepsy. Therefore, one of the most important findings of this study was that two blood markers were independently associated with refractory epilepsy.

Despite recent advances in epilepsy treatment, current interventions and AEDs are not effective in more than 30% of patients. In recent years, the potential role of some naturally unfolded proteins that can form pores in cell membranes and contribute to the irregular ion flows that characterize electrical discharges has attracted attention to resistance to treatment. Ion channels are considered the main target in understanding the mechanism and treatment of epilepsy. It is suggested that the etiology and pathophysiology of acquired epilepsy are related to the formation of de novo ion channels under new conditions, probably from naturally unfolded proteins. One of these proteins, αS, is a small protein expressed predominantly in CNS neurons β-Lactam antibiotics such as ceftriaxone that reduces the frequency of seizures by binding to α-synuclein and preventing its aggregation [12].

Serum αS levels have been generally investigated in Parkinson's disease. However, there are very few studies on epilepsy. Serum αS levels have been reported to be significantly increased in children with epilepsy and correlate with disease severity [13]. In a study, serum αS levels did not show a statistically significant difference between epilepsy patients and healthy volunteers [14]. Another study determined that serum and cerebrospinal fluid (CSF) αS levels were increased in children with refractory epilepsy compared to those with non-refractory epilepsy. It was stated that the increase in both CSF and serum was associated with αS crossing the blood-brain barrier after epilepsy [15]. It has been suggested that the mechanisms regulating αS expression predispose this protein to aggregation and that this protein increases membrane permeability by forming protofibrils or pores that serve as ion channels. Transcriptional modification of α-synuclein, such as methylation/demethylation

(epigenetic mechanisms) of regulatory regions in the α-synuclein gene, controls the level of αS expression. Since αS aggregation is concentration-dependent, it may be affected by its expression level. The increase in expression level also increases the formation of αS oligomers and aggregates. Again, post-translational modification of αS, such as phosphorylation, predisposes α-synuclein to conformational changes and aggregation and may affect its pore-forming capacity [12].

According to the transport hypothesis, in drug-resistant epilepsy, overexpression of drug efflux transporters in the BBB causes decreased uptake of AEDs into the brain, resulting in resistance to treatment [16]. The role of P-gp in neurological, neuroinflammatory, and neurodegenerative diseases, including Alzheimer's disease and epilepsy, is increasingly recognized [17]. Overexpression of P-gp in the BBB is known to be one of the mechanisms underlying resistance to antiepileptic drugs in patients with epilepsy. However, knowing which AEDs or which substances are substrates for P-gp is important in managing the treatment of patients with refractory epilepsy.

It has been reported that there is an increase in P-gp activity, especially in the brain tissue of drug-resistant temporal lobe epilepsy (TLE) patients [18]. In their study on adult patients with drug-resistant TLE, Asadi-Pooya and Sperling [19] applied the P-gp inhibitor verapamil in addition to AEDs at different doses. Seizure freedom was observed in the patient group given higher doses of P-gp inhibitor.

An experimental study investigated the effect of the P-gp inhibitor verapamil on the transfer of phenobarbital, lamotrigine, and felbamate to the brain and reported significantly increased concentrations of the three AEDs in the brain. Thus, it was concluded that overexpression of P-gp in epileptic tissue probably limited the access of these AEDs to the brain [20]. It has also been suggested that for certain AEDs, the efficiency of substrate recognition or transport by P-gp may differ between species and even between tissues [21].

In this study, P-gp levels were determined to be higher in patients with resistant epilepsy compared to children with treatment-responsive epilepsy but this increase was statistically insignificant. Patient-specific factors, drugs, and an insufficient number of patients may have affected the results.

ADA has a function in regulating extracellular adenosine concentration and central nervous system functionality. Adenosine is a purine nucleoside that can be released during neuron damage and produced intracellularly or extracellularly by nucleotide dephosphorylation. It crosses the cell membrane following its concentration gradient. Adenosine has anticonvulsant properties, which are mainly mediated by the activation of adenosine A1 receptors. The antiepileptic role of adenosine is based on the fact that activation of A1 receptors reduces glutamate release and glutamatergic sensitivity and hyperpolarizes neurons [22]. Studies have reported that inhibiting adenosine deamination effectively reduces seizures in various epileptic animal models [23, 24].

It has been reported that the increase in ADA and adenosine kinase activity may cause a decrease in adenosine levels, even leading to the progression of epileptogenesis and epilepsy [25,26]. Neuroprotective mechanisms based on the inhibition of neuronal excitability through adenosine were reported to be effective in reducing intracellular calcium levels and nerve damage [10]. In this study, the low ADA activity in the non-drug-resistant group can be interpreted as antiepileptic drug treatment suppressing the increase in ADA activity. Adenosine is an endogenous modulator of brain functions that offers anticonvulsant properties. Its levels may also increase during nerve damage. The high level of ADA activity in children with refractory epilepsy may be due to the release of adenosine as a result of damage following seizures. The increase in ADA activity may be characterized by a vicious cycle that increases the deamination of adenosine, resulting in a decrease in adenosine levels and triggering a new seizure.

The limitations of this study are cross-sectional design, small sample size and data from a single center.

CONCLUSIONS

The present study is the first to investigate serum ADA activity in refractory epilepsy. In addition to clinical features, the use of ADA and αS as biochemical risk factors in the follow-up of patients with epilepsy may be useful. Nevertheless, these results need to be confirmed by prospective studies with many patients.

ABBREVIATIONS

ADA – adenosine deaminase activity
AEDs – anti-epileptic drugs
ANOVA – One-way analysis of variance
αS – α-synuclein
AUC – area under the curve
BBB – blood-brain barrier
CSF – cerebrospinal fluid
EEG – electroencephalogram
ILAE – International Association Against Epilepsy
P-gp – p-glycoprotein
ROC – receiver operating characteristic
TLE- temporal lobe epilepsy

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AUTHORS' CONTRIBUTION

NŞ – participated in the design of the study, data collection, data analysis, review of the literature, writing the original draft reviewing it for important intellectual content and searching for references.

DBK – conceptualization, methodology, formal analysis, writing the original draft preparation, visualization, searching for references and validation.

AG – coordination of the study and participants, organizing and planning of tasks, participating in the design of the study, writing and reviewing the draft.

MC – data acquisition, analysis, and interpretation; writing the original draft and reviewing it for important intellectual content.

HP – data acquisition, analysis, and interpretation; writing the original draft.

HG – participated in data collection, review of the literature, and writing of the draft.

SM – conceptualization, methodology, writing the original draft and reviewing it for important intellectual content.

All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

None to declare.

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