Original article:

Features of folate cycle disorders in children with ASD

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Abstract:

Aim: to show the effect of genetically determined folate cycle deficiency in children with autism spectrum disorders (ASD).***Participants:*** 89 children (57 boys; 32 girls, Ukraine, 2-10 years old); participants were diagnosed with ASD. The control group consisted of 34 children with ASD.***Diagnostic methods:***polymerase chain reaction (PCR), complex immunological research, diagnosis of infection, determination of biomarkers.***Results and discussion:***Hyperhomocysteinemia was revealed in 87% of cases ($p < 0.05$; $Z < Z0.05$).The indicated form of immunodeficiency was noted among 91% participants in the study, while only in 27% children of the control group had a similar immunological phenotype.The serum concentration of folic acid was increased in 64%, and reduced in 21% of cases. An increase of vitamin B12 also occurred in 64%, and vitamin B6 - only in 43% of cases.

Keywords: folate cycle, autism spectrum**,** vitamin B12, cytomegalovirus infection

Bangladesh Journal of Medical Science Vol. 19 No. 04 October'20. Page : 737-742
DOI: https://doi.org/10.3329/bjms.v19i4.46634

Introduction

In recent decades, ideas about the genetic heterogeneity of autistic spectrum disorders in humans have become firmly established[1]. Frye R.E. in a recent fundamental review of this problem, he considers enzymes of folate cycle and mitochondrial disorders as the basis for autistic spectrum disorders in children[2,3]. Moreover, mutations of Nitric oxide synthases (NOS) are observed as the cause of autistic disorders in some families[4,5]. Nevertheless, the rare causes of autism spectrum disorders are increasingly being described.

Polymorphisms of folate cycle enzymes are considered as a significant reason for thegenetic locationto the development of autistic disorders[6,7]. In addition, polymorphisms of genes involved in thefolic acid cycle are associated with hyperhomocysteinemia, cardiovascular catastrophes[8] and the later stage of dementia[9], congenital anomalies, primarily the nervous system[5,10], and pathology in pregnancy, including multiple spontaneous abortions[11], a high risk of cancer developing, including colon and rectal[12], due to a DNA methylation[13-15].

There are few reports of autism in adults and children suffered from Neuroinfectious diseases, mainly the opportunistic infections[10,16]. The folic acid cycle is realized with three key enzymes: methylenetetrahydrofolate reductase (MTHFR), methionine synthase reductase (MTRR) and methionine synthase (MTR)[17]. This cycle functions along with the methionine cycle, and as a result the homocysteine is synthesized. Such metabolite has a toxic effect on the vascular endothelium, causing endothelial dysfunction, and CNS neurons, induce synaptic imbalance and cell death.

The goal is to identify the effect of genetically determined folate cycle deficiency in children with autism spectrum disorders (ASD).

Materials and methods

Participant

The sample consisted with 89 children (57 boys; 32 girls, Ukraine, 2-10 years old); all participants were

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diagnosed with ASD. The control group consisted of 34 children with ASD; they were of the same age and gender.

Diagnostic Methods

Polymorphisms of folate cycle genes were detected by polymerase chain reaction (PCR) in three centres: Neurological Research Institute (USA), Kharkiv Specialized Centre of Medical Geneticsand medical laboratory "Synevo". All patients underwent a comprehensive immunological examination at Department of Clinical Immunology and Allergy of Bogomolets National Medical University. The examination included a general blood count, the study of lymphocytes subpopulation using laser-based flow cytometer(Epics XL Flow Cytometer, USA) and indirect immunofluorescence method with monoclonal antibodies CD (Beckman Coulter, United States). Phagocytosis was determined using a latex fixation test, by the number of active phagocytes, myeloperoxidase activity (MPO) (flow cytometer) and NADPH oxidase (Nitro Blue-Tetrazolium test (NBT)). Immunoglobulin concentrationwas determined by the Radial immunodiffusion (RID) or Mancini method.The concentration of IgE, IgD classes and IgG subclasses (IgG1, IgG2, IgG3, and IgG4) were measured by Enzyme linked immunosorbent assay ("Vector-BEST", RF). In addition, the evaluation of a PCR-based assay for the detection and species identification of herpesviruses(herpes type 1 and 2, varicella-zoster virus, Epstein-Barr virus, cytomegalovirus, HHV-6 and 7) measles and rubellaare presented. Serological tests were also performed using ELISA to identify virus-specific IgM and IgG antibodies in serum. All children underwent conventional brain MRI(T1- and T2-weighted, FLAIR) on 1.5 Tesla Magnetic Resonance Imaging Scanners.

Statistical Data Analysis

Statistical analysis of the data obtained was performed using structural and comparative analysis by the electronic program Microsoft Excel. The reliability of difference scores has been studied by **Student'st-test** (parametric criterion) and **coordination number Z**by Urbach curve (non-parametric criterion). The correlation between polymorphisms of folate cycle and immune status was studies withPearson Chi-square ($\chi 2$) criterion. The obtained value was compared to the table with given degree of freedom and confidence intervals p = 0.05 and p = 0.01. **Yates's correction** was additionally applied for actual values from 5 to 9, and **Fisher's exact test** for less than 5. The calculation formula is given below:

$$X^2 = \sum_{i=1}^{r} \sum_{j=1}^{c} \frac{(O_{ij} - E_{ij})^2}{E_{ij}} \quad (1)$$

Research results and discussion

Genetic research

The genetic testing results indicate that the majority in the group of sick children had 2-4 polymorphisms in the folate cycle enzyme (88% of cases), and the ratio of heterozygous and homozygous states can be represented as 1.2 to 1.0. Only in 12% of cases patients had one polymorphism, mainly in a homozygous state (Table 1). Generally, those children also had other genetic disorders that affect their mental development, namely Leigh syndrome (1 case), Rett syndrome(1 case) and DSN (1 case), as well as hemochromatosis (1 case) and mutations in NOS (2 cases). Also,the substitution at nucleotide of the MTHFR gene prevailed (56% of cases), however, but no relationswith genetic disorder was noted.

Table 1The structure of the study group (n = 89) by the number of polymorphisms of the folate cycle enzyme

The number of mutations	Patients with this mutation (in %)
1	12
2	36
3	39
4	13

Biomarker analysis

Biomarkers analysis in the group of sick children showed the presence of hyperhomocysteinemia in 87% of cases ($p < 0.05$; $Z < Z0.05$), however, there was a significant fluctuation of this metabolite, depending on the child's diet. Serial studies were needed for several weeks to form the correct judgment on the presence of hyperhomocysteinemia. The serum concentration of folic acid was increased in 64%, and reduced in 21% of cases. An increase of vitamin B12 also occurred in 64%, and vitamin B6 - only in 43% of cases. Indeed, hyperhomocysteinemia was the most significant biochemical marker for determining thefolate cycle disorders (Fig. 1). Screening for total homocysteine (tHcy) in blood may identify candidates for appropriate genetic examination among children with autism spectrum disorders. However, in some cases, the normal content of homocysteine did not exclude the presence of several polymorphisms in the folate cycle.

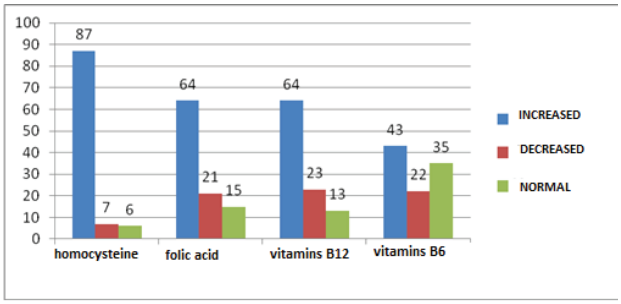

Figure1 The serum concentrations of the studied biomarkers among children with folate cycle disorder (n = 89)

Assessment* of the *microbial load

Patients of the study group were diagnosed with abnormally frequent, prolonged, severe viral infections caused by opportunistic microorganisms that usuallylead to complications. Congenital cytomegalovirus infection with central nervous system damage was registered in 17% of cases (Fig. 2). The diagnosis was confirmed by PCR analysis of either cerebrospinal fluid or serum, and in some cases by specific IgM. Generally, those patientswere diagnosed with cerebral palsy due to motor disorders, although with a deeper analysis, signs of autism spectrum disorders were additionally noted. Deficits in mental abilities have been reported at birth. Autism is often combined with intellectual disorders.

Subacute sclerosing panencephalitis (SSPE) caused by measles and / or rubella viruses is registered in 21% of cases, usually without an initial period of typical rashes. These progressive lesions of CNS were caused both by the infections and live attenuated vaccine against measles, rubella and mumps. Depending on the current stage of the pathological process, children with such neurological lesions were observed with isolated ASD of short duration or severe CP. In addition, children had an initial period of normal development and regression in ASD shortly after infection. Generally, the first signs of regression were recorded 2-4 weeks after vaccination, but the natural infection usually remained unidentified due to the absence of exanthema. The diagnosis was confirmed with persistence of specific IgM or an abnormally increased of IgG, which was hundreds and thousands of times higher of normal.

Human lymphotropic herpesviruses(CMV, EBV, HHV-6, HHV-7) were the most common manifestation of infection (78% of cases). The diagnosis was confirmed by PCR analysis of serum. Many children suffered from mononucleosis-like illness[18].

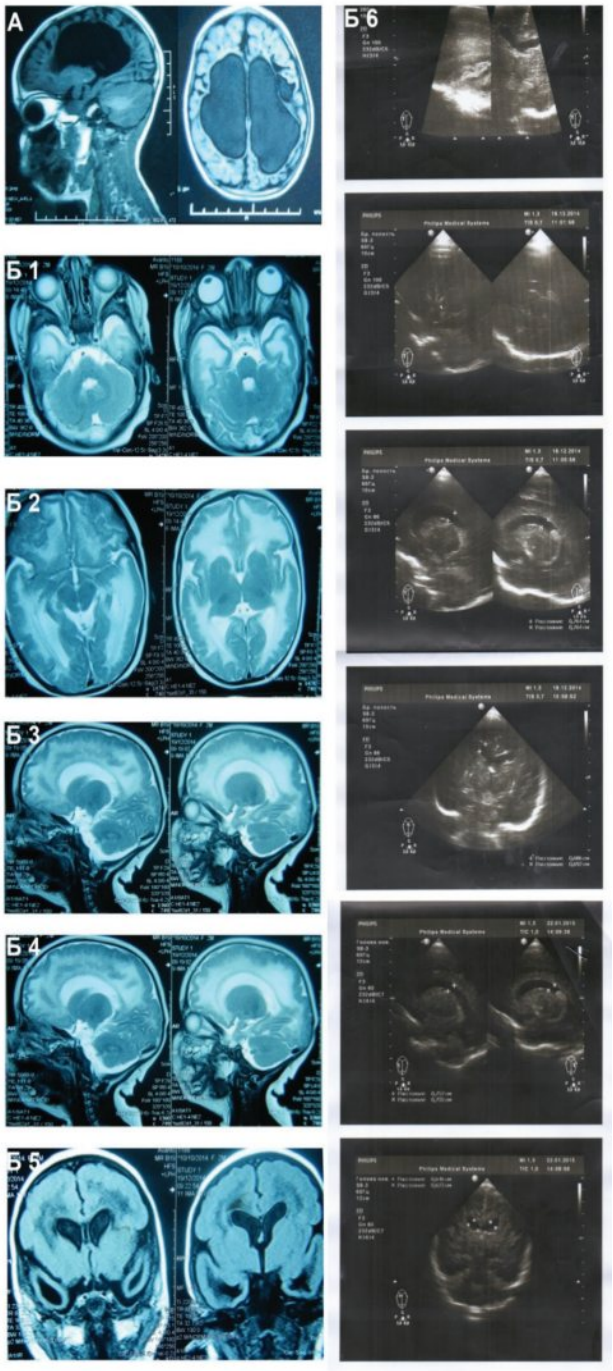

Figure2 Signs of congenital cytomegalovirus infection in children with genetic disorders of the folate cycle (А - CNS malformations with ventriculomegaly and hypogenesis of the cerebral hemispheres, probably due to virus infection in early ontogenesis; Б - a complex of common signs of CMV infection in late pregnancy: at temporal lobes (1), leukoencephalopathy (2, 3,4), agenesis of corpus callosum(5), ventriculomegaly (2, 3, 4) and lenticulostriate vasculopathy(6); own observations)

12% of children were observed with regression in ASD after a seriousrotavirus infection.The onset of regression in ASD occurred in only 4% of children who overcame influenza. There were registered 32% cases of recurrent respiratory tract infections. The diagnosis was confirmed by a microbiological method. Chronic candidiasis was diagnosed in 17% of cases (Fig. 3).

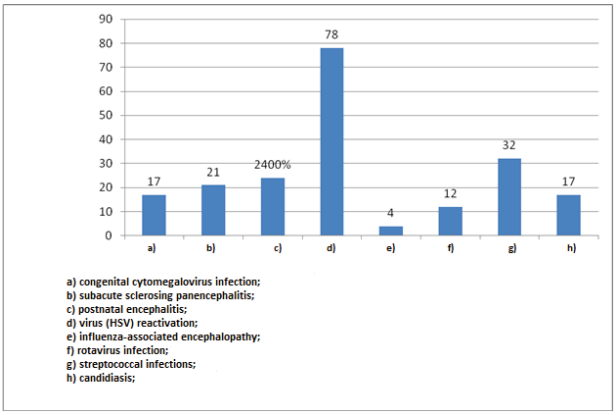

Figure3 The structure of the infections in patients with the folate cycle disorder (n = 89)

Viral infections registered among children of the study group were a damaging factor inducing ASD.

Assessment of immune status

Almost all children with folate cycle disorder were determined as immunocompromised individuals, and some similar immunity disorders were also noted. The basis of the identified immunodeficiency was a sharply reduced number of lymphocytes with the CD3 – CD16 + CD56 + phenotype, called natural killers (NK), and the CD3 + CD16 + CD56 + phenotype, called natural killer T-cell(NKT) in peripheral blood. These minority subpopulations are extremely important in the implementation of antiviral and antitumor immunity.The indicated form of immunodeficiency was noted among 91% participants in the study, while onlyin 27% children of the control group had a similar immunological phenotype. The immune system disordersandthe immune status were less significantamong children with ASD: a decrease in the number of CD8 + T-lymphocytes (23%), CD4 + T-cells (12%), CD19 + B-lymphocytes (9% of cases). Thus, in only 23% of cases there was a total deficit of all the main antiviral subpopulations of lymphocytes(NKT-cells, NK and NKT-lymphocytes). Meanwhile such children were registered with the highest viral load at the beginningof study. Predominant was a deficiency in NK cellsof innate immunity, and the number of CD8 + T-lymphocytes often increased, which contributed to a certain decrease in the viral load on the child's body. This form of immunodeficiency could be easily identified in a general blood test by a decreased number of large granular lymphocytes. Only one in ten participants in the study group had a total decrease in all the studied lymphocyte subpopulations, as blood count reveals lymphocytopenia. Violations were noted in the humoral immunity.43% of children were diagnosed with dysimmunoglobulinemia with immunoglobulins subclass deficiency, either isolated or combined, but it was most often shallow and transient.

Based on immune status analysis, the main immunological phenotypes in children with a folate cycle disorders can be distinguished[15]. The main immunological phenotype was NK and / or NKT cells deficiency. This phenotype was observed in almost all children in the study group. 62% of children hada sharp decrease in the number of lymphocytes in both subpopulations. The isolated NK cell deficiencieswere rare - in 13% and 16% of cases, respectively, and the total deficiency of antiviral cells of innate and adaptive immunity was detected only in 23% of cases, i.e. less than 1/3 (Fig. 4).

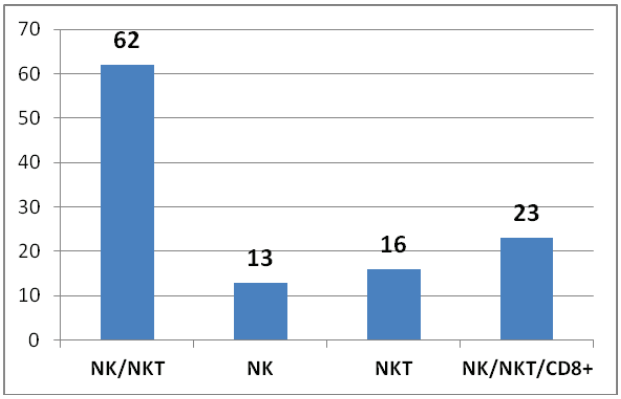

Figure4 The structure of antiviral immunity violations in children with folate cycle disorder (n = 89)

The results of Pearson Chi-square ($\chi 2$) criterion for assessing correlation between polymorphisms of folate cycle and immune status are given in table 2. According to table. 2, there is statistically significant association of the studied genetic disorders with a deficit of cytotoxic T-lymphocytes, expressed**CD8 receptor**, and a deficiency of the microbicidal enzyme of myeloperoxidase phagocytes ($p = 0.05$).

Table 2The results of Pearson Chi-square (χ2) criterion for assessing the deficiency of NK / NKT cells when comparing patients of the studied (n = 89) and control (n = 34) groups.

Cell / Factor Deficiency	χ^2	Statistical significance *
NK and / or NKT-cells	51.1	p=0.01
NK- cells	27.2	p=0.01
NKT- cells	23.1	p=0.01
CD8+ T- lymphocytes	4.6	p=0.05
CD4+ T- lymphocytes	2.7	Statistical significance
Myeloperoxidase	4.4	p=0.05
IgM	2.1	Statistical significance
IgG	2.9	Statistical significance
IgA	3.1	Statistical significance
IgE	3.1	Statistical significance
Hypoimmunoglobulin	3.3	Statistical significance
Dysimmunoglobulinemia	37	Statistical significance

*Note. * - χ2 = 3.841 at p = 0.05 and 6.635 at p = 0.01*

Nevertheless, isolated NKdeficiency occurred in 19% of cases, since more than half of the participants in the study group had an expanded phenotype (54%). Meanwhile, along with a pronounced and persistent deficiency of NK and NKT-cells less profound and predominantly transient disorders were noted in other parts of the immune system, namely, deficiency of CD8 + T-lymphocytes, various types of disimmunoglobulinemia and deficiency of myeloperoxidase. This, the clinical picture of the infections could be modified. If resistance to NK and NKT-cells is reduced and the resistance to intracellular microorganisms (which determines the development of opportunistic infections) decreases, then in case of dysimmunoglobulinemia, recurrent respiratory tract infections were additionally recorded. They were usually caused by Streptococcus pneumoniae and Staphylococcus aureus. At the same time, recurrent candidiasis occurred due to myeloperoxidase deficiency. In the case of NK and NKT cell deficiency along with hypoimmunoglobulinemia, a phenotype of variable immunodeficiency was observed (17% of cases), and also those children were registered with bacterial infections, such as pneumonia, pyelonephritis and septicemia. It must be emphasized that in every tenth case a phenotype resembling severe combined immunodeficiency was recorded due to the accumulation of lymphopenia and hypo- or dysimmunoglobulinemia (Fig. 5). Such children suffered from congenital CMV with severe malformations of CNS or viral encephalitis with severe residual symptoms. These were children diagnosed with CP, although with a deeper analysis, they had signs of an ASD. Generally, there were 4 polymorphisms of the folate cycle in their genome.

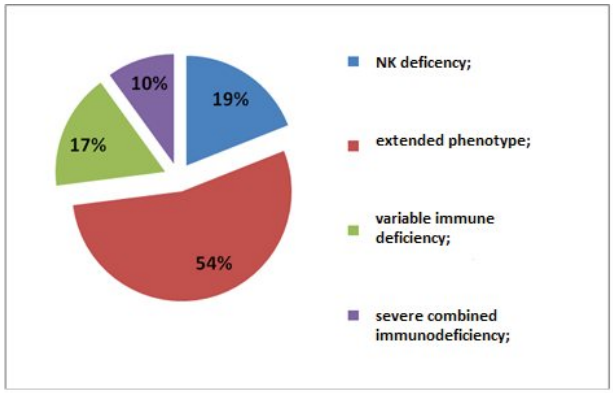

Figure5 The structure of immunological phenotypes in children with folate cycle (n = 89)

According to the above data, it can be assumed that the folate cycle leads to the development of a special immunodeficiency, in which NK and NKT cells can drastically reduce resistance to intracellular microorganisms, tumours, and a tendency to develop autoimmune reaction and delayed type hypersensitivity.

Conclusions

The study results show that the cause of immunodeficiency in children with folate cycle disorder. The main immunological phenotype was NK / or NKT cell deficiency. Almost all children with ASD had this phenotype. 62% of children had a sharp decrease in the number of lymphocytes in both subpopulations.

Source of fund: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest: The author declare that there is no competing interests.

Ethical clearence: The research was approved by ethical committee of O'Bogomolets National Medical University.

Authors's contribution:

Data gathering and idea owner of this study : DmitryMaltsev

Study design: DmitryMaltsev

Data gathering: DmitryMaltsev

Writing and submitting manuscript: DmitryMaltsev

Editing and approval of final draft: DmitryMaltsev

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