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ORIGINAL ARTICLE

The importance of the blood levels of homocysteine, folic acid and vitamin B12 in children with malignant diseases

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Summary

Purpose: *Hyperhomocysteinemia is associated with carcinogenesis. Since only little research exists on hyperhomocysteinemia and malignancy in children, the possible relationship between homocysteine and childhood malignancies remains unknown. The aim of the present study was to determine the serum levels of homocysteine, folic acid and vitamin B12 in children with malignant and benign tumors prior to therapy (surgical treatment and/or chemotherapy), and after treatment of malignant diseases as well.*

Methods: *Forty-six children with newly diagnosed malignant diseases (solid tumors and lymphoproliferative/myeloproliferative (LP/MP) malignancies) and 6 children with benign tumors were included in the present study. The patient age ranged between 2 months and 18 years.*

Results: *Significantly increased homocysteine concentrations were identified in children with malignant diseases compared with those with benign tumors ($p<0.01$). The plasma concentration of homocysteine in children with malignant diseases decreased significantly following treatment ($p<0.05$). Before treatment, the concentration of folic acid in children with malignant solid tumors was significantly higher than in children with malignant LP/MP diseases ($p<0.01$). Following treatment, the concentration of folic acid was significantly decreased ($p<0.05$) in children with malignant solid tumors, while it was not significantly increased in children with malignant LP/MP diseases ($p>0.05$). The concentration of vitamin B12 in children with malignant diseases (solid tumors and LP/MP diseases) increased significantly following treatment ($p<0.01$), while it increased substantially ($p<0.01$) in patients with solid malignancies following treatment.*

Conclusion: *Homocysteine could be a marker of malignancy in chidren. Further research is needed to establish the importance of homocysteine, folic acid and vitamin B12 in pediatric malignant diseases.*

Key words: *children, folic acid, homocysteine, malignancy, vitamin B12*

Introduction

Malignant diseases represent the second most common cause of death in children [1]. A better understanding of the pathogenesis and development of malignant disease is necessary to develop more effective treatments and, ultimately, cures [2].

Carcinogenesis is the progressive, gradual transformation of a normal cell into a malignant cell, under the influence of genetic alterations [3,4]. Recent studies have demonstrated that cancer is not solely a genetic disease [5]. Currently, malignant diseases are considered to be a result of genetic alterations in conjunction with epigenetic alterations [6]. A prime example of epigenetic alteration is DNA methylation [7].

Each DNA methylation can be associated with carcinogenesis [8-10]. There are two reported mechanisms of DNA methylation that are usually associated with malignancy - DNA hypomethylation and uracil misincorporation into DNA [10-12]. Homocysteine is a sulfur amino acid and a normal intermediate in methionine metabolism

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[13]. Homocysteine metabolism consists of the intersection of two pathways: remethylation to methionine, which requires folic acid and B12 coenzymes; and transsulfuration to cysteine, which requires pyridoxal-5'-phosphate, the B6 coenzyme. Homocysteine metabolism is included in both mechanisms of DNA methylation.

Hyperhomocysteinemia is associated with many diseases including vascular and neurodegenerative disorders, autoimmune diseases, congenital anomalies, diabetes, kidney dysfunction, osteoporosis, neuropsychiatric diseases and malignant diseases [14]. Additionaly, hyperhomocysteinemia is a biological marker of oxidative stress and represents a high risk for endothelial damage [15,16].

Because comprehensive research into the importance of homocysteine in children with malignant diseases, especially solid malignant tumors, has not yet been conducted, the present prospective study was undertaken to assess the significance of homocysteine in this population group. Furthermore, the aim of the present study was to assess the blood levels of homocysteine, folic acid and vitamin B12 in children with malignant diseases and benign tumors, and to determine whether the levels of these parameters changed following therapy.

Methods

Patients

In the period between January 2009 and January 2011, 46 children with newly diagnosed malignant diseases and 6 children with bening tumors were included in this study that was performed at the Institute of Mother and Child Health Care of the Republic of Serbia "Dr Vukan Cupic''. The patient age ranged from 2 months to 18 years, with the majority (30.8%) falling between 13 and 18 years of age. Children with malignant diseases were classified into two groups: solid tumors and LP/MP diseases. In children with solid tumors, the diagnosis was histopathologically confirmed with either tumor biopsy or tumor extirpation, whilst in patients with LP/MP malignancies, the diagnosis was confirmed by immunohistochemistry and molecular diagnostics.

The present prospective study was approved by the Ethics Committee of the Faculty of Medicine, University of Belgrade (Republic of Serbia), and conducted in accordance with ethical standards.

Biochemical measurements

Venus blood samples (3-4 ml), were taken in the morning before breakfast and collected in test tubes with EDTA for homocysteine assessment and in test tubes without anticoagulant for vitamin B12 and folic acid assessment (2-3 ml). Blood samples were centrifuged and frozen at -20 ^{0}C within 1 h after venipuncture, and kept frozen until assayed. The ACL Elite Pro device (Instrumentation Laboratory, USA) was used to identify homocysteine by automated latex-enhanced immunoassay for the quantitative determination of total L-homocysteine in human citrated plasma. A conversion factor of 1.17 (homocysteine EDTA vs homocysteine citrate) was used according to the manufacturer's instructions.

Serum vitamin B12 and folic acid levels were simultaneously determined by chemiluminescent detection technology (Access Immunoassay System, Beckman Coulter, USA), in accordance with the manufacturer's recommendations.

Plasma concentrations of homocysteine and serum concentrations of vitamin B12 and folic acid were measured prior to any treatment, as well as after therapy (surgical treatment and/or chemotherapy) in children with malignant diseases.

Statistics

The statistical analysis was performed with SPSS 10.0 for Windows. Results were expressed as means (X) ± standard deviation (SD). After checking data distribution, the appropriate statistical tests (x^2, Student's t-test or ANOVA) were used. A p value <0.05 was considered as statistically significant.

Results

Demographic, clinical and developmental histogenesis in patients with malignant diseases (N=46) are shown in Table 1. The types of solid tumors and LP/MP malignancies are shown in Table 2. Organ pathology and localization of malignant diseases are presented in Table 3. Demographic, clinical and pathological characteristics of the group of patients with benign tumors (N=6) are shown in Table 4. The comparisons of plasma level of homocysteine, and serum levels of folic acid and vitamin B12, before therapy and between the groups of patients with malignant diseases or benign tumors are displayed in Table 5.

Statistical analysis of the results before therapy, and taking into consideration the total number of patients with malignant diseases (both groups, solid tumors and LP/MP diseases) and those with benign tumors showed that plasma levels of homocysteine differed significantly ($p<0.05$). However, there was no significant difference in serum levels of folic acid and vitamin B12 between these two groups (Table 5).

The levels of homocysteine, folic acid and

Table 1. Demographic, clinical and pathological characteristics in the group of patients with malignant diseases

Characteristics	*N (%)*					
Gender						
Male	25 (54)					
Female	21 (46)					
Age (mean) at diagnosis						
Years (range)	7.16 (0.16-17.91)					
Malignancy						
Solid tumors						
Total	29 (63)					
Males	13 (52)					
Females	16 (76.2)					
LP/MP						
Total	17 (37)					
Males	12 (48)					
Females	5 (23.8)					
Age of appearance/type of malignancy						
Age, years/range	0-1	2-3	4-6	7-9	10-12	13-18
Solid tumors	5 (17.2)	9 (31)	2 (6.9)	2 (6.9)	2 (6.9)	9 (31)
LP/MP	0 (0)	3 (17.6)	0 (0)	3 (17.6)	6 (35.3)	5 (29.5)
Histogenesis						
Ectoderm derived	12 (26.1)					
Endoderm derived	5 (10.9)					
Mesoderm derived	27 (58.7)					
Multilineage differentiation	2 (4.3)					
Increased tumor markers (not determined in all cases)						
AFP	12 (50)					
β-HCG	1 (16.7)					
VMA and HVA (urine)	6 (37.5)					

LP/MP: lymphoproliferative malignancies/myeloproliferative diseases, VMA: vanillyl mandelic acid, HVA: homovanillic acid, AFP: alpha fetoprotein, β-HCG: beta-human chorionic gonadotropin

Table 2. The types of solid tumors and lymphoproliferative malignancies/myeloproliferative diseases in the group of patients with malignant diseases

Tumor type	*N (%)*
Solid tumors	29 (63)
Neuroblastoma	8 (17.4)
Primitive neuroectodermal tumor	3 (6.5)
Adrenocortical carcinoma	1 (2.1)
Wilms tumor	4 (8.7)
Rhabdomyosarcoma	5 (11)
Osteosarcoma	1 (2.1)
Hepatoblastoma	2 (4.3)
Yolk sac tumor	2 (4.3)
Embryonal testis carcinoma	1 (2.1)
Immature teratoma	1 (2.1)
Malignant schwannomas	1 (2.1)
Lymphoproliferative malignancies/ myeloproliferative diseases	17 (37)
Hodgkin lymphoma	1 (2.1)
Non- Hodgkin lymphoma	2 (4.3)
Acute lymphoblastic leukemia	8 (17.4)
Acute myelogenous leukemia	2 (4.3)
Chronic myelogenous leukemia	1 (2.1)
Myelodysplastic syndrome	2 (4.3)
Histiocytosis	1 (2.1)

Table 3. Pathology and localization of malignant diseases (N=46)

Characteristics	*N (%)*
Solid tumor	29 (63)
Lymphoproliferative malignancies/ myeloproliferative diseases	17 (37)
Urinary system organs	4 (8.7)
Genital system	10 (21.7)
Nervous system	9 (19.6)
Locomotor system	4 (8.7)
Endocrine system	1 (2.2)
Lymphatic tissue and organs	2 (4.3)
Bone marrow	14 (30.5)
Digestive system	2 (4.3)

Table 4. Demographic clinical and pathological characteristics in the group of patients with benign tumors (N=6)

Characteristics	*N (%)*
Gender – male/female	50/50
Mean age at surgery, years (range)	3.25 (1.30-6.50)
Hematoma	1 (16.6)
Ganglioneuroma	1 (16.6)
Morgagni's hydratid	1 (16.6)
Cystic nephroma	1 (16.6)
Myofibroblastic tumor	1 (16.6)
Fibrous inflammatory pseudotumor	1 (16.6)

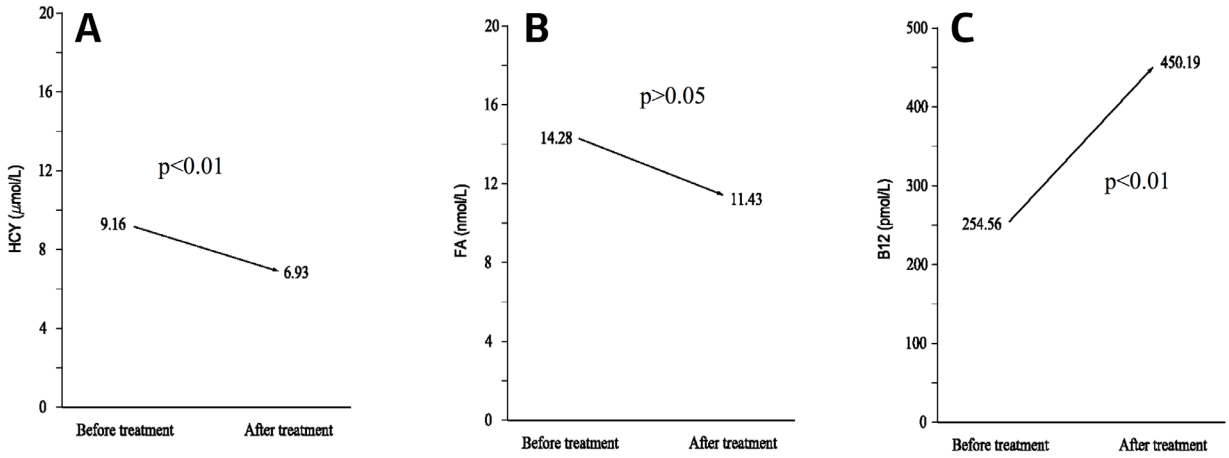

Figure 1. Differences in levels of **A)** homocysteine (HCY), **B)** folic acid (FA) and **C)** vitamin B12 in patients with malignant diseases (both groups taken i.e. solid tumors and lymphoproliferative malignancies/ myeloproliferative diseases) prior to and after therapy.

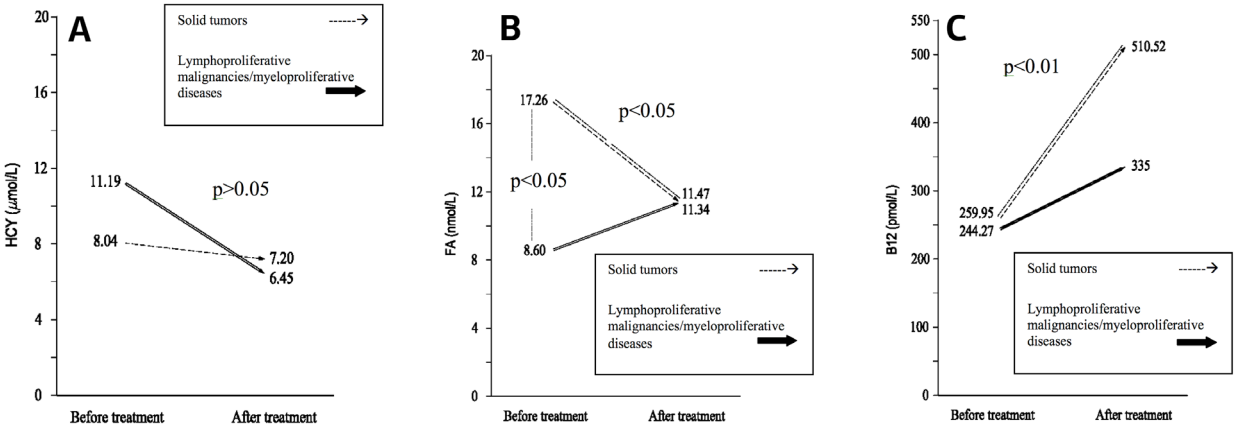

Figure 2. Comparison of the levels (mean values) of **A)** homocysteine, **B)** folic acid and **C)** vitamin B12 before and after therapy according to type of malignant disease (solid tumors vs lymphoproliferative malignancies/ myeloproliferative diseases) .

vitamin B12 were measured prior to and after therapy in both groups of patients with malignant diseases.

The applied therapy included surgery (61.5%) and chemotherapy (78.8%). The level of homocysteine was significantly lower after therapy in patients with malignant diseases (malignant solid tumors and patients with LP/MP diseases; p<0.01; Figure 1A). On the contrary, no significant difference in the levels of folic acid in patients with malignant diseases measured prior and after therapy was detected (Figure 1B; p>0.05). The level of vitamin B12 was significantly higher after therapy in patients with malignant diseases (solid tumors and LP/MP diseases; p<0.01; Figure 1C).

Changes in the levels of homocysteine, folic acid and vitamin B12 were further analysed in subgroups, i.e. before and after therapy in the group of patients with malignant solid tumors compared with the group of patients with LP/MP diseases (Figure 2). Figure 2A shows no statistical difference in the levels of homocysteine before therapy as well as after therapy between the groups of patients with solid tumors and LP/MP diseases (p>0.05). Before therapy the levels of fo-

Table 5. Comparisons of plasma levels of homocysteine, and serum levels of folic acid and vitamin B12, before therapy, and between the groups of patients with malignant disease or benign tumors (X±SD)

Parameter	*Malignant disease*	*Benign tumor*	*p-value*
HCY (μmol/L)	9.84±5.84	4.13±2.34	<0.01
FA (nmol/L)	14.1±10.91	15.61±5.08	>0.05
B12 (pmol/L)	292.57±220.47	381.02±120.75	>0.05

HCY: homocysteine, FA: folic acid

lic acid were statistically different between these two groups of patients ($p<0.05$). After therapy the levels of folic acid in patients with malignant solid tumors were significantly lower ($p<0.05$), while in the group of patients with LP/MP diseases no statistical difference was detected ($p>0.05$) (Figure 2B). Before therapy the levels of vitamin B12 did not differ significantly between the two investigated groups ($p>0.05$). After therapy in the group of patients with malignant solid tumors the levels of vitamin B12 were significantly increased ($p<0.01$), while in the group of patients with LP/MP diseases no statistically significant difference was detected ($p>0.05$) (Figure 2C).

Discussion

The wide range of molecular diversity in malignant diseases in children indicates that many complex interactions are involved in their etiology. The significance of homocysteine and its cofactors, folic acid and vitamin B12, in the etiology of malignancy in children has not yet been clarified. Although some research has been performed, there is still not enough information to firmly establish a link between hyperhomocysteinemia and malignancy in children [17-23]. To date, it has been established that hyperhomocysteinemia is associated with acute lymphoblastic leukemia and lymphoma [18,19], as well as specific subtypes of pediatric brain tumors [20]. It has also been established that folate intake during pregnancy significantly reduces the risk of acute lymphoblastic leukemia in early childhood [22].

The present study sought to determine the diagnostic and prognostic relevance of homocysteine, folic acid and vitamin B12 levels in children with malignant diseases. Our research has shown that the plasma concentration of homocysteine in children with malignant diseases is significantly higher than in patients with benign tumors. On the other hand, folic acid and vitamin B12 concentrations were not significantly different between these two groups. Previous research involving children with acute leukemia has demonstrated increased demands for folic acid in leukemia, resulting in reduced serum folate concentration and reduced catabolism and folate excretion [18]. Homocysteine levels have been considered to be a sensitive indicator of folate status. Because folate deficiency is often associated with hyperhomocysteinemia it has been concluded that adequate folate intake can reduce homocysteine levels in adults [16]. The results of recent case-control epidemiological studies indicated that increased levels of homocysteine is a risk factor and biological marker for several types of tumors in adults [24-27]. However, the increase in plasma homocysteine concentration has not been correlated with folic acid and vitamin B12 deficiency, which has led to the hypothesis that hyperhomocysteinemia is most probably associated with tumor progression [24,27], and that there is an important correlation between an increase in plasma homocysteine and tumor growth [28,29]. Therefore, homocysteine can not be considered as a marker of cell damage only. The results of our study suggest the possibility that homocysteine is a marker of tumor-associated disorders in children, and that it can be used as a marker in the differential diagnosis of malignant and benign tumors in children. Our claim could be of particular value in the pathology of malignant tumors in children because there are currently no sufficiently specific and sensitive tumor markers for many malignancies in children.

The present research has demonstrated that there are no significant differences in folic acid and vitamin B12 concentrations between children with malignant diseases and those with benign tumors. Serum folic acid concentration in patients with LP/MP diseases was significantly lower than that of patients with solid tumors, mostly because of different needs of folic acid for *de novo* synthesis of nucleotide precursors. More specifically, it is assumed that the high-affinity transport system for folic acid is excessively active in leukemic cells and, therefore, leukemic cells in patients contain a higher folate concentration than non-leukemic cells.

The ratio between folate in leukocytes and serum is significantly increased [30].

After the patients with malignant diseases were treated (surgery and/or chemotherapy) the tumor mass was supposed to be reduced, which was accompanied by a significant drop in the plasma concentration of homocysteine. This finding confirms the possibility that the plasma concentrations of homocysteine are associated with defective metabolism of homocysteine in tumor cells and with the level of tumor progression; therefore it is a potentially useful tumor marker, not only in differential diagnosis but also in monitoring for therapy efficacy. There have been no statistically significant changes in the serum levels of folic acid following treatment of malignant diseases (both solid tumors and LP/MP diseases). However, a review of the results in certain groups

of malignant diseases (solid and hematological) in our study has shown the following changes: a significant drop in folic acid concentration in the group with solid tumors, and an increase, not statistically significant, in the group with LP/MP diseases. This drop in folic acid concentration in patients with solid tumors is extremely unusual; previous research on malignancies in adults has confirmed an inverse relationship between homocysteine and folic acid levels [31-33].

Homocysteine metabolism pathway has a pivotal link with ''one-carbon metabolism''. The resulting hyperhomocysteinemia is one of the indicators of impaired methylation capacity and could lead to a number of pathological processes. Some authors have found a link between homocysteine concetration and tumor growth [31], whereas others believe homocysteine manifests its carcinogenic effect by entering the metabolic pathways of other compounds [32].

In other studies, a link between hyperhomocysteinemia and some cancers in adults has been clearly established. It has been shown that increased homocysteine concentrations in adults are associated with colorectal cancer [8,9,11,12,33], cervical cancer [34], breast cancer [35], pancreatic cancer [36] and laryngeal cancer [37].

Following treatment, patients with malignant diseases (both solid tumors and LP/MP diseases) showed a significant increase in serum vitamin B12 levels. This increase was more evident in the group of patients with solid tumors compared with those with LP/MP diseases. The importance of this finding is currently unknown because the assessment of serum vitamin B12 has limited sensitivity and specificity in diagnostic applications [38].

Conclusion

Based on existing data, it is still not possible to precisely define the degree to which hyperhomocysteinemia contributes to carcinogenesis, and to which degree hyperhomocysteinemia and carcinogenesis are associated with low serum levels of folic acid and vitamin B12 or genetic predisposition. In general, the role of increased plasma level of homocysteine in children is still a matter of debate in clinical practice due to the absence of adequately-powered, large-scale clinical studies. Our results suggest that increased plasma levels of homocysteine are associated with malignancy in children, and, therefore, homocysteine may be used as a diagnostic and/or prognostic tumor marker. It seems that homocysteine could be a metabolic marker for the monitoring of tumor activity rather than the cause of tumorigenesis.

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