The relationship between cerebrospinal fluid osteopontin level and central nervous system involvement in childhood acute leukemia

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The aim of this study was to evaluate the relationship between cerebrospinal fluid (CSF) osteopontin (OPN) levels and central nervous system (CNS) involvement in children with a diagnosis of acute leukemia. The study sample consisted of 62 patients who had been diagnosed with acute leukemia. The control group consisted of 16 patients that had presented and had no malignant disease, CNS infection or chronic disease. CSF OPN levels were studied with enzyme-linked immunosorbent assay (ELISA) method. The mean CSF OPN level was 32.76±49.22 ng/ml in the patients at the time of diagnosis and 14.93±6.84 ng/ml in the control group ($p>0.05$). The mean CSF OPN level was 27.68±32.73 ng/ml at the time of diagnosis in the group without CNS involvement and 53.48±89.21 ng/ml in the group with CNS involvement ($p>0.05$). However, the CSF OPN level at the time of CNS relapse in patients who developed CNS involvement during follow-up (127.4±52 ng/ml) was significantly higher than in the group without CNS involvement at diagnosis and follow-up (mean CSF OPN level: 27.68±32.73 ng/ml) ($p<0.001$). The analysis of CSF OPN levels at the time of diagnosis-before relapse and at the periods of relapse and remission in patients who had CNS involvement at diagnosis and/or follow-up revealed statistically significant differences between the time points ($p<0.001$). High CSF OPN levels in childhood acute leukemia patients may be used as evidence for CNS involvement, and any increases found in CSF OPN levels may be a preliminary predictor for CNS involvement.

Key words: acute leukemia, central nervous system, cerebrospinal fluid, osteopontin.

Acute leukemia is the most common malignant disease in children. It accounts for 25-30% of childhood cancers, and its incidence in childhood is 30-40 per million[1]. A study from our country has reported the frequency of acute leukemia as 34.9% within childhood malignancies[2].

With the start in the 1970's of using risk-focused intensive chemotherapy regimens according to the clinical and laboratory features, the success rate of acute leukemia treatment has increased[3]. The five-year eventless survival rate in children has reached 80-90%[4]. Despite this great success, central nervous system (CNS) relapses are currently one of the greatest obstacles to full recovery[5]. Current clinical studies have focused on effective treatment methods to decrease the CNS relapse rate in leukemia patients. Accordingly, methods for the early diagnosis of CNS leukemia have started to attract the interest of investigators.

Osteopontin (OPN) is secreted by many cells such as activated T cells, natural killer (NK) cells, macrophages, Kupffer cells, kidney-breast-

skin epithelial cells, the nervous system and vascular smooth muscle cells, and tumor cells. In the bone marrow, OPN is predominantly synthesized and secreted by the osteoblasts and to a lower degree by pre-osteoblasts, osteocytes and other hematopoietic cells. The secreted form of OPN is widely distributed in body fluids (blood, urine, bile, and milk)[6-8]. With its arginine-glycine-aspartate structure, it binds to the other integrins in the bone matrix during resorption, is regulated by local cytokines, and acts as a bridge between cells and minerals as a molecule with an acidic, phosphorylated glycoprotein structure. It has characteristic functional structures. It is able to interact with CD44 α, β integrin receptors thanks to these structures and plays a role in events such as cell-matrix and cell-cell signalization[9]. It is encoded by a single gene found in chromosome 4q13. It may show differences according to cell type and condition with post-translational modification, and isoforms with molecular weights varying between 41 and 71 kda have been detected. OPN effects change according to its various isoforms, multiple receptors and binding regions[10]. OPN migration has been shown to increase extracellular matrix invasion. OPN levels have been shown to increase in many cancers and especially in metastatic ones, and this is related to an aggressive course and unfavorable prognosis of the disease[6,11].

Studies on OPN are generally in adult solid tumors. However, a few studies on hematological malignancies in recent years have provided hope that OPN may be used as a diagnostic and prognostic marker[12-15].

The aim of this study was to determine the relationship between cerebrospinal fluid (CSF) OPN levels and CNS involvement in patients with a diagnosis of acute leukemia and whether OPN could be used as an early marker in the early period when signs and symptoms of CNS involvement have not yet appeared.

Material and Methods

This was a prospective study to determine the relationship between CSF OPN levels and CNS involvement in patients who received a diagnosis of acute leukemia at Dr. Sami Ulus Obstetrics and Pediatrics Training and Research Hospital (Dr. SUCH), Pediatric Oncology Clinic between March 2008 and June 2010 and to find out whether OPN could be used as an early marker in the periods when signs and symptoms of CNS involvement have not yet appeared. Ethics committee consent was obtained from the Dr. SUCH ethics committee.

Characteristics of the Study Population

The study sample consisted of 62 patients who had received a diagnosis of acute leukemia between the above-mentioned dates. For children diagnosed with acute leukemia, consent was obtained from the families. Approximately 2 ml was separated from the CSF samples that were obtained as part of the treatment protocol before intrathecal treatment was administered and/or if there were signs of CNS involvement; samples were kept at -30°C and registered. The presence of $5x10^6$/L or more blasts in the CSF was evaluated as CNS involvement. Patients who were included in the study were divided into two groups as those with and without CNS leukemia at the time of diagnosis and/or during the follow-up. There were 13 patients with CNS involvement and 49 patients with no CNS involvement in the study group. The CSF samples of these patients were included in the study as follows: (1) The CSF samples of patients who had no CNS involvement during diagnosis and follow-up were included in the study (a total of 49 CSF samples); (2) There was CNS involvement at the time of diagnosis in 3 of the 13 patients with CNS involvement. The remaining 10 developed CNS relapse while their treatment and follow-ups continued; (3) The CSF samples of 3 patients who had CNS involvement at the time of diagnosis and the 6 samples that were obtained after CNS remission was obtained were included in the study; and (4) We also included the CSF samples at the time of diagnosis, before relapse, and during the relapse and remission periods for the 10 patients who developed CNS relapse while being followed under treatment. However, one patient died before entering remission, and we were therefore unable to obtain a remission

CSF sample. One patient had no pre-relapse CSF sample. One patient was transferred from another center with CNS relapse and died quickly, and we therefore only have one sample from the relapse period (a total of 35 CSF samples).

The pre-relapse CFS samples were obtained a median of 58 days previously (46-90 days previously) as they were taken before intrathecal treatment according to the administered treatment protocol. The CSF samples during remission were taken a median of 32 days later (28-40 days). We therefore included the 90 CSF samples of a total of 62 patients with this method. The information of the patients was screened from archived information.

The control group consisted of 16 children from the same age group as our study group who had presented at the Dr. SUCH emergency service and general outpatient departments, had no malignant disease, CNS infection, or chronic disease, and who underwent lumbar puncture (LP) to investigate the cause of a febrile convulsion or fever. A total of 16 CSF samples belonging to these patients were studied. The clinical and laboratory findings of the patients at the time of diagnosis are shown in detail in Table I.

Of the 13 patients that developed CNS leukemia at the time of diagnosis and/or during follow-up, only 4 of the patients had symptoms at the time of CNS involvement. One patient had personality change, 2 had headaches, and 1 had headache, vomiting and diplopia. The only neurological sign on physical examination was strabismus in 1 patient. The CNS involvement diagnoses in the other patients were made by evaluating the CSF samples that had been obtained during the diagnosis or before the routine administration of intrathecal treatment. The median levels of CSF cell, protein and glucose were 40/mm^3 (5-5200), 42 mg/dl (14-522) and 62 mg/dl (0-140), respectively.

In the control group, the female/male ratio was 7/9 and the age range was 1-132 months. The median age in this group was 14 months. A LP had been performed in 11 patients to determine the cause of fever and in 5 patients because of complicated febrile convulsion. None of the patients had laboratory findings consistent with CNS infection and none had growth on the CSF culture.

CSF OPN Level Determination Method

The CFS OPN levels were analyzed at Düzen Laboratory using the IBL brand ready-to-use kit (Kit no: 27158) for the enzyme-linked immunosorbent assay (ELISA) method as financed by the Helping Children with Cancer Association (KANCODER) on the CSF samples that had been stored protected from light at -30°C.

Statistical Evaluation of the Data

Statistical evaluation was performed with the Statistical Package for Social Sciences (SPSS) 16 package software, while the nonparametric longitudinal data were analyzed with the LD-

Table I. Clinical and laboratory findings at the time of diagnosis of patients

	Patients without CNS involvement (n: 49)	Patients with CNS involvement (n: 13)
Male/Female	28/21	9/4
*Age (months)	84 (19-180)	78 (30-180)
*White blood cell count (/mm^3)	6200 (720-407000)	13800 (2000-216000)
*Hemoglobin value (g/dl)	8.2 (2.7-13.4)	9.8 (4.5-16.2)
*Thrombocyte count (/mm^3)	55000 (3000-616000)	90000 (8000-618000)
Immunophenotypes		
Acute lymphoblastic leukemia	43	12
Acute myeloid leukemia	4	1
Mixed leukemia	2	0

*Median

F1 design at the Ankara University Medical Faculty, Department of Biostatistics. The Mann-Whitney U test and chi-square test were used for categorical data. A p value less than 0.05 was considered as statistically significant for the difference between the groups for the tests used.

Results

The 62 patients with acute leukemia included in this study consisted of 49 with no CNS leukemia at the time of diagnosis or during follow-up and 13 patients that comprised the group who had CNS leukemia at the time of diagnosis and/or during the follow-up. There was no statistically significant difference with respect to age, gender, hemoglobin (Hb), white blood cell (WBC), and thrombocyte (Plt) values for these two groups ($p>0.05$). When the blast morphological type and immunophenotypic features at the time of diagnosis were compared, there was no statistically significant difference between the groups ($p>0.05$).

One of the 62 acute leukemia patients included in the study was transferred to our clinic while being followed-up with CNS relapse at another center, so we were only able to measure the OPN levels in the CSF samples at the time of diagnosis of 61 acute leukemia patients. Three patients who had CNS involvement at the time of diagnosis are included in the 61 patients for whom the CSF OPN levels at the time of diagnosis could be studied. The CSF OPN level range at the time of diagnosis for these patients was 1-300 ng/ml, with a mean value of 32.76±49.2 ng/ml. The control group CSF OPN levels ranged from 5-27 ng/ml, with a mean value of 14.93±6.8 ng/ml. There was no statistically significant difference between the two groups ($p=0.651$).

The CSF OPN levels at the time of presentation for the group with no CNS involvement at the time of diagnosis and during follow-up ranged from 1-153 ng/ml, with a mean value of 27.68±32.73 ng/ml, while these values were 1-300 ng/ml and 53.48±89.21 ng/ml, respectively, for the group that had CNS leukemia at the time of diagnosis and/or during follow-up. In the second group, 3 of 13 patients had CNS involvement at the time of presentation. There was no statistically significant difference between the two groups ($p=0.502$). There was no statistically significant difference between the mean CNS OPN levels of the two groups at first presentation and the mean CNS OPN level of the control group ($p=0.807$ and $p=0.371$).

The CNS OPN level during CNS relapse in the 10 patients who had no CNS leukemia at the time of presentation and developed CNS relapse during the follow-up ranged from 39.5-236 ng/ml, with a mean value of 127.4±52 ng/ml. There was a very statistically significant difference between the mean CNS OPN levels of this group and the control group ($p<0.001$), and there was also a very statistically significant difference between CNS mean OPN levels at the time of presentation of this group and the group that did not develop CNS leukemia at the time of diagnosis or during follow-up ($p<0.001$).

The CSF OPN level range was 1-161 ng/ml, with a mean value of 42.4±44.2 ng/ml, at the time CNS remission was achieved in 8 of the 10 patients (2 patients died without CNS remission) who developed CNS relapse during follow-up and in the 3 patients who had CNS involvement at presentation. This value was still statistically significantly higher than the mean CSF OPN level of the control

Table II. CSF OPN values of patients with CNS involvement by time point

Evaluation time point of patients with CNS involvement	n	OPN (ng/ml) Mean±SD (ranges)
At diagnosis	12	53.48±89.21 (1-300)
Before relapse	8	29.51±17.50 (4-58)
At relapse	10	127.40±52.00 (39.5-236)
At remission	11	42.40±44.20 (1-161)

group (p=0.003).

When the LD-F1 design used to analyze nonparametric longitudinal data was employed to evaluate the CFS OPN levels at the time of presentation-before CNS relapse, at the time of relapse and at CNS remission periods in the 13 patients who had CNS involvement at first presentation and/or during follow-up, the difference between these time points was statistically very significant (p<0.001) (Table II, Fig. 1).

This study has shown that the CSF OPN level in acute leukemia cases starts to increase before the known clinical and laboratory findings of CNS involvement appear in the group with CNS involvement. It elevates even further to form a peak when these findings appear, then starts to decrease when the CNS involvement is treated.

Discussion

The uneventful survival rate has increased to 80% in childhood acute lymphoblastic leukemia (ALL) thanks to intensive and multiple chemotherapy administration, while the frequency of relapse in the CNS, testis or other extramedullary locations has decreased significantly. However, CNS relapses remain one of the biggest obstacles to full recovery[5]. Determining patients prone to CNS relapses is important in determining the treatment. Hyperleukocytosis, T cell immunology, the Philadelphia chromosome, and t (4;11) type genetic changes in children are known to be associated with an increased CNS relapse risk. The Berlin-Frankfurt-Münster (BFM) group has shown in their evaluation of patients between 1995-1999 that a traumatic LP at the time of diagnosis increased the CNS relapse total risk from 3.5% to 8%[16-18]. The polymorphism of genes related to methotrexate metabolism is thought to be a possible risk factor. Although the biological cause of leukemia cell development is unknown, their predisposition for the CNS is recognized. It has been postulated that these blasts can continue to exist in the subarachnoid veins without being affected by chemotherapy and can return to the bone marrow to cause bone marrow relapse. It is known that high-dose methotrexate administration and treatment protocols such as CNS radiation directed towards the CNS prevent CNS leukemia and also bone marrow relapses[5]. However, despite all these advanced treatment methods, CNS involvement in ALL cases is seen at the rate of about 6% and has a quite unfavorable effect on the prognosis. Recent studies have focused on effective treatment methods to decrease CNS relapse rates in leukemia patients. Methods directed towards the early diagnosis of CNS leukemia have therefore also become a point of interest for investigators.

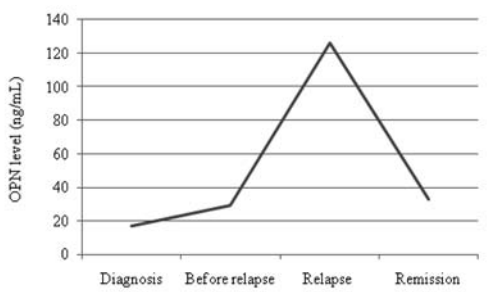

Figure 1. LD-F1 design of CSF OPN values by time point in patients with CNS involvement.

Although there are many methods to diagnose CNS leukemia, the standard method is cytocentrifugation of the CSF and evaluation under light microscopy for the presence of leukemic cells. CSF cytology is considered the gold standard for diagnosis, but it is possible to have false-negative or false-positive results[19]. In conditions causing pleocytosis, such as radiotherapy, recurrent intrathecal interventions and chemical reaction, it can be difficult to morphologically differentiate cells present in the CSF from blasts. The tdt stain (immunocytology) can help in differentiating leukemia cells in such cases[20].

Magnetic resonance imaging (MRI) has been shown to be highly sensitive in demonstrating meningeal pathology. However, it can produce false-positive results following diagnostic or therapeutic LP. A study has revealed that immunocytology is more sensitive then MRI (89% to 44%) in the diagnosis of B-lineage

ALL[20].

Advanced studies in recent years have focused on flow cytometry and polymerase chain reaction to increase sensitivity. Hegde et al.[21] have shown that multicolor flow cytometry can detect blast cells present at a rate of even 0.2% of total lymphocytes by using multiple antibody panels for B and T cell antigens. A new study in 60 patients with CNS involvement has shown that flow cytometry has twice the diagnostic value of cytomorphology.

A more recent study has shown that high expression of interleukin (IL)-15 in childhood leukemia is meaningful in the diagnosis of CNS leukemia and later relapse. It is thought that the cytokine has a pathogenetic role in leukemic cell migration to the CNS[22,23].

In addition, increased levels of adhesion molecules such as L-selectin and beta-2-microglobulin, soluble CD27 and CD9 in the CSF have been reported to be markers that can be used in the early diagnosis of CNS involvement[24-28].

There has been an increase recently in the number of studies investigating the relationship between OPN and tumor metastases and an aggressive clinical course, especially in adult malignancies. This study was performed to determine the relationship between the CSF OPN level and CNS involvement in pediatric patients with acute leukemia. Of the 62 acute leukemia patients included in the study, 57 had ALL and 5 acute myeloid leukemia (AML). Two of the 57 patients with ALL (3.5%) had CNS involvement at the time of diagnosis, and the WBC count of these patients at this time was 26000/mm^3 and 152000/mm^3, consistent with the literature. One of the 5 patients with a diagnosis of AML (20%) had CNS involvement at the time of diagnosis, with a WBC count at the time of presentation of 127000/mm^3. The high rate of CNS involvement in patients with a diagnosis of AML is secondary to the low number of patients. The patients that did not have CNS leukemia at the time of diagnosis and developed CNS relapse during follow-up all had a diagnosis of ALL. The lack of a statistically significant difference between this group and the group that did not have CNS leukemia at the time of the diagnosis or during follow-up regarding age, gender, Hb, WBC, Plt, and blast immunophenotypic features may be due to the different numbers of subjects in the groups.

There are no data in the literature regarding the CSF OPN level of healthy individuals. However, there are two studies on the CSF OPN level in multiple sclerosis (MS) and one study on the CSF OPN level in teratoid/rhabdoid tumors (AT/RT). Braitch et al.[29] compared the CSF OPN levels in three groups with MS, non-inflammatory neurological disease or non-MS other neurological disease. The groups with MS and other inflammatory neurological disease had significantly higher CSF OPN levels compared to the control group with non-inflammatory neurological disease. The mean CSF OPN level of the control group was 457 (SD: 271) ng/ml. Chowdhury et al.[30] studied two groups with MS or other neurological disease, and found the CSF OPN levels to be 0.6-32 mcg/ml. Kao et al.[31] compared the CSF OPN levels in three groups with CNS AT/RT, medulloblastoma or nonmalignant disease (hydrocephaly, epilepsy). The median CSF OPN level was 1175 ng/ml (859.5-1599) in the AT/RT group, 524.5 ng/ml (456-607.5) in the medulloblastoma group, and 168 ng/ml (145.3-211.8) in the non-malignant group.

The CSF OPN level has been reported to increase in many neurological diseases, and we therefore chose our controls from subjects with no known chronic neurological disease. To the best of our knowledge, this is the first study in the literature demonstrating the CSF OPN levels of individuals in the pediatric age group with no neurological disorder.

We did not find a difference between the mean CSF OPN levels of the acute leukemia patients at the time of diagnosis and the control group. Of the 62 acute leukemia patients included in the study, only 3 had CNS leukemia at the time of diagnosis, and this may be the reason why no difference was found. The mean CSF OPN level at the time of relapse in the 10 acute leukemia patients who developed CNS relapse during follow-up was significantly higher than

the mean CSF OPN level of the acute leukemia patients who had no CNS involvement at the time of diagnosis or during follow-up. Figure 1 shows that there was a significant increase in the CSF OPN levels from a median of 58 days ago and the values at the time of relapse in each of the 10 patients who developed CNS relapse. It is also interesting that the CSF OPN levels of these patients showed a significant decrease at the time of remission compared to the values at relapse. These findings indicate that the CSF OPN level starts to increase before relapse in these patients, reaches a peak at the time of relapse, and decreases when the CNS remission has been achieved. This difference between the time points was also found to be statistically significant. It is therefore thought that an increase in the CSF OPN levels can be an indicator for CNS involvement that will soon appear.

This study to determine the relationship between CNS involvement and CSF OPN levels in childhood acute leukemia patients is, to our knowledge, the first in the literature on this subject. We have found that the presence of high OPN levels in childhood acute leukemia may indicate CNS involvement and may even be used as a pre-marker before the involvement. However, as there are no previous similar studies in the literature, this matter needs to be studied with larger series and together with serum OPN levels.

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