Significance of soluble CD40 and CD40 ligand levels in childhood acute lymphoblastic leukemia patients

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ABSTRACT

Aim: Acute lymphoblastic leukemia (ALL) is the most prevalent malignant disorder in childhood. CD40 is a member of the tumor necrosis factor (TNF) receptor family. Soluble CD40 (sCD40) was previously found to be associated with poor prognosis in adult patients with hematologic malignancies such as acute myeloid leukemia (AML) and multiple myeloma. Experience in children, however, is rather limited. The present study aimed to investigate the significance of sCD40 and sCD40 ligand in children with ALL.

Material and Method: This study recruited 44 children treated at Erciyes University, Division of Pediatric Hematology & Oncology between February 2008 and February 2010. We investigated the relationship between sCD40/sCD40 ligand at the diagnosis and remission during continuation phase with the prognosis of children with ALL. We also considered the data on treatment response, relapse, and outcome.

Results: The participating patients (20 girls and 25 boys) were between 22 months-18 years (mean 7.6±4.6 years) and had leukocytes at diagnosis between 870-7,416,660/mm³ (median 15,150/mm³). Thirty-five patients were diagnosed with B, whereas nine were diagnosed with T phenotype. In this cohort, 13 patients were classified in the standard-risk (SR) group, 20 patients were in the intermediate-risk (IR) group, and 11 patients were categorized in the (HR) group according to the Turkish Acute Lymphoblastic Leukemia Berlin Frankfurt Munich (TR-ALL BFM) protocol. The serum levels of CD40/CD40 ligand at diagnosis were 22.41±9.91 ng/ml and 15.17±5.49 ng/ml, respectively, whereas these levels at remission were 0.22±0.38 ng/ml and 1.04±0.51 ng/ml, respectively. We detected significant changes in CD40 and CD40 ligand levels (p=0.008 and p<0.005, respectively) and early response on the 8th day. Although not significantly correlated with sCD40, the final outcome had a significant relationship with early response detected on the 8th day of treatment. We found the cut-off value of sCD40 to be 28.15 ng/dL in our cohort. The effect of sCD40 on event-free survival was clinically significant, but it did not yield statistical significance.

Conclusion: Overall, our findings suggest that sCD40 is measured as increased at diagnosis of childhood ALL. On the basis of its physiological effect, sCD40 may have a role in modulating antitumor response in pediatric ALL and be a useful prognostic marker.

Keywords: Acute lymphoblastic leukemia, child, CD40, CD40 ligand, prognosis

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most prevalent malignant disease in childhood. While treatment response in ALL would not see 10% in the early 1960s, complete recovery can now be achieved in about 80-90% of patients thanks to multiple chemotherapies (1-3). Previous research identified many important prognostic factors that enable identifying patients in different treatment groups. Thanks to the treatment by risk detected in the patient, children with a higher risk of relapse would be subjected to more intensive treatment, while those with a lower risk of relapse would be treated with protocols covering reduced or free toxic chemotherapies (e.g., cranial radiation and anthracyclines) (4-7).

CD40 is a member of the tumor necrosis factor (TNF) receptor family and is expressed in all developmental stages of B cells, monocytes, macrophages, platelets, follicular dendritic cells, and eosinophils. Apart from blood cells, CD40 is synthesized in kidney, keratinocyte, synovial membrane fibroblast, and skin fibroblast cells,
thymus epithelial cells, and active endothelial cells (8,9). Being in the form of a trimer, CD40L (CD154), the ligand of CD40, is a member of the TNF family and is synthesized in active CD4 positive T cells. Besides, it may appear on different levels of monocytes, active B cells, vascular endothelial cells, smooth muscle cells, dendritic cells, and platelets. The CD40-CD40L relationship was first demonstrated in T-cell-dependent B-cell immunity. Recent research highlighted that this receptor-ligand relationship also plays an important role in atherosclerosis and various inflammatory events. Apart from its trimeric membrane-bound form, CD40 L may appear in an 18-kDa soluble (sCD40L) form (10-12).

Contemporary literature reports that sCD40 and sCD40L levels are measured high at diagnosis and can be independent indicators in determining the prognosis in adult patients with chronic lymphocytic leukemia (CML), acute myeloblastic leukemia (AML), and multiple myeloma (12). Ultimately, the present research aimed to investigate whether serum sCD40 and sCD40L levels have an impact on prognosis in childhood ALL patients.

**MATERIAL AND METHOD**

**Patient Recruitment**

We planned to carry out the study with ALL patients who were treated and followed up at Erciyes University, Faculty of Medicine, Division of Pediatric Hematology & Oncology between February 2008 and February 2010. Accordingly, the sample was composed of 44 patients with ALL and treated using the Turkish Acute Lymphoblastic Leukemia Berlin Frankfurt Munich (TR-ALL BFM 2000) protocol. We then studied sCD40 and sCD40L levels in the patients’ blood samples at diagnosis and during the remission (maintenance therapy) and explored their associations with treatment risk group, sex, age, leukocyte count at diagnosis, lactate dehydrogenase (LDH), erythrocyte sedimentation rate (ESR), immunophenotype, central nervous system (CNS) involvement, treatment response, relapse, and survival.

This was prospective research conducted at Erciyes University, Faculty of Medicine, Division of Pediatric Hematology & Oncology. While funded by the Turkish Society of Hematology Research Projects Commission, our research was granted ethical approval by the Ethics Committee of the mentioned faculty of medicine (No: 2010/120 dated 10.08.2010).

**ALL Treatment Protocol**

The treatment protocol consisted of induction, consolidation (protocol M), re-induction, and maintenance therapies. Prednisolone, vincristine, daunorubicin, L-asparaginase (L-ASP), cytarabine, 6-mercaptopurine (6-MP), and cyclophosphamide are used, and intrathecal methotrexate (MTX) is applied in induction therapy. Protocol M, on the other hand, includes 6-MP, high dose MTX (1 or 5 g/m²) given four times, and intrathecal MTX. Dexamethasone, cytarabine, vincristine, doxorubicin, L-ASP, cyclophosphamide, and 6-mercaptopurine are utilized in re-induction therapy. If the patient falls in the high-risk group, they may receive high-risk (HR) block therapy following induction therapy. HR block therapy includes dexamethasone, vincristine, high-dose cytarabine and MTX, L-ASP, cyclophosphamide, daunorubicin, vindesine, ifosfamide, and etoposide. The overall treatment is complemented to 2 years with weekly MTX and daily 6-MP in maintenance therapy (13).

**Collecting and Storing Blood Samples**

We obtained 2 ml peripheral blood samples from the patients. Following coagulation at room temperature, the samples were centrifuged at 300g for 5 minutes at +4°C to elute the sera. After collecting the sera, they were centrifuged at 10,000g for 10 minutes, and the upper parts were separated and stored at -70 °C.

**Studying sCD40/sCD40L with Enzyme-Linked Immunosorbent Assay (ELISA)**

We prepared the washing solution at a ratio of 1/10 using the concentrated washing solution (1 ml) and distilled water (9 ml) in the test kit. Biotin and streptavidin conjugates used in the analysis were prepared during incubation. For this purpose, the test buffer, biotin, and streptavidin conjugates were mixed 1/100 in separate tubes.

We used positive control solutions of the test kit for positive controls. The ELISA kit and its contents, stored at 2-8°C, were brought to room temperature prior to the analyses. We designated the plate plan of the eight-well ELISA kit as the positive control in the first well, negative controls in the second and third wells, and samples in the subsequent wells, respectively. 20 μl of sample sera were added to each of the control and sample wells, and the plates were covered and incubated at room temperature (18-23°C) for 2 hours. At the end of the incubation, we washed the plates three times using the pre-prepared washing solution. Then, the wells were filled with 100 μl of conjugate liquid, covered, and incubated for 1 hour at room temperature. At the end of the incubation, the washing protocol was repeated for each plate. We added 25 μl of streptavidin conjugate to the wells and incubated covered for 10 minutes at room temperature. The reaction was
terminated by adding 100 μl of stop solution to each well at the end of the incubation. Finally, we measured absorbances using a spectrophotometer at a wavelength of 450 nm.

**Calculations and Evaluation**

We used an ELISA plate reader (ELX 800 Absorbance Microplate Reader, Bio-Tek Inst., Inc.), programmed to absorbance values at 450 nm wavelength, for measurements. We divided the sum of the absorbance values of the negative controls in the experiment by the number of negative controls and reached the cut-off value by multiplying the result with the F value of 2.5. Then, the absorbance values of the samples were compared with the cut-off value. Samples with an absorbance value equal to or higher than the cut-off value were considered positive.

**Statistical Analysis**

We referred to the Shapiro Wilk test to explore the normal distribution. While the normally distributed data were expressed as means and standard deviations, non-normally distributed data were shown as medians (minimum-maximum). The pairwise comparisons were performed using independent samples t-test and Mann-Whitney U test. We compared the data of more than two groups using the Kruskal-Wallis test and Chi-square test.

Receiver Operating Characteristic (ROC) curve was utilized to determine the impacts of sCD40 and sCD40L levels on prognosis. Sensitivity and specificity were calculated based on standard formulas. The cut-off value of sCD40 was identified to determine the early response to treatment (day 8) by ROC analysis. Then, we performed a Kaplan-Meier survival analysis to compare the event-free survival (EFS) rates of groups with a value above and below this cut-off value. The log-rank test was referred to compare survival findings. All statistical analyses were performed using the “Statistical Package for Social Sciences” (SPSS 15.0) program, and a p-value <0.05 was considered statistically significant.

**RESULTS**

We carried out this study with a total of 44 patients (19 females and 25 males) aged between 22 months and 18 years (mean age=7.6±4.6 years) who were diagnosed with ALL and treated with the TRALL BFM protocol at Erciyes University, Faculty of Medicine, Division of Pediatric Hematology & Oncology between February 2008 and February 2010. Thirty-five patients were diagnosed with B, whereas nine were diagnosed with T phenotype. According to the risk classification of the TR-ALL BFM protocol, 13 patients were categorized in the standard-risk (SR) group, 20 patients were included in the intermediate-risk (IR) group, and 11 patients were placed in the high-risk (HR) group. Considering phenotypes, the patients in the SR group were B ALL. While 16 of 20 patients in the IR group were B ALL, the remaining were T ALL. Of the 11 patients in the HR group, six were B ALL, whereas five were T ALL (Table 1). CNS involvement was detected in 6 patients at diagnosis. While four patients with CNS involvement were in the IR group, two were in the HR group. We could not detect CNS involvement in any of the patients in the SR group.

<table>
<thead>
<tr>
<th>Risk group</th>
<th>N</th>
<th>Age (min-max)</th>
<th>Sex</th>
<th>Immunophenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR</td>
<td>13</td>
<td>3.6±1.4 years (22 months-72 months)</td>
<td>8 girls, 5 boys</td>
<td>13 B</td>
</tr>
<tr>
<td>IR</td>
<td>20</td>
<td>3.6±1.4 years (27 months-17 years)</td>
<td>6 girls, 14 boys</td>
<td>16 B, 4 T</td>
</tr>
<tr>
<td>HR</td>
<td>11</td>
<td>9±4.1 years (18 months-18 years)</td>
<td>5 girls, 6 boys</td>
<td>6B, 5 T</td>
</tr>
<tr>
<td>Total</td>
<td>44</td>
<td>8.9±5.4 years (22 months-18 years)</td>
<td>19 girls, 25 boys</td>
<td>35 B, 9 T</td>
</tr>
</tbody>
</table>

The patients had leukocytes at diagnosis between 740/mm³ and 7,416,600/mm³ (median=15,150/mm³), and absolute neutrophil count at diagnosis between 80/mm³-20,300/mm³ (median=600/mm³). While hemoglobin level at diagnosis distributed between 2.2 g/dL-13.9 g/dL (median=7.3 g/dL), the patients had platelets at diagnosis between 3,000/mm³-409,000/mm³ (median=53,000/mm³). The LDH levels of the patients at diagnosis varied between 172 u/l and 5,133 u/l (median=400 u/l), and the ESR at diagnosis varied between 3 mm/hr and 120 mm/hr (median=26 mm/hr) (Table 2).

<table>
<thead>
<tr>
<th>Median (min-max)</th>
<th>Diagnosis (n=44)</th>
<th>Remission (n=31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocyte (/mm³)</td>
<td>15,150 (870-741,660)</td>
<td>6,240 (2,530-9,930)</td>
</tr>
<tr>
<td>Absolute neutrophil (/mm³)</td>
<td>600 (80-20,300)</td>
<td>3,210 (1,360-6,670)</td>
</tr>
<tr>
<td>Hemoglobin (gr/dl)</td>
<td>7,3 (2.2-13.9)</td>
<td>12.9 (10.8-15.3)</td>
</tr>
<tr>
<td>Platelets (/mm³)</td>
<td>53,000</td>
<td>266,000</td>
</tr>
<tr>
<td>ESH (mm/h)</td>
<td>26 (3-120)</td>
<td>6 (2-31)</td>
</tr>
<tr>
<td>LDH (u/l)</td>
<td>400 (172-5133)</td>
<td>240 (181-424)</td>
</tr>
</tbody>
</table>

In this cohort, 9 (20%) patients developed relapse during the five-year follow-up, but we detected relapse in only one patient in the next five years. While only medullary relapse was detected in 6 of all patients, we discovered both medullary relapse and CNS involvement in 3
patients. Only one patient demonstrated isolated CNS relapse. While 9 out of 10 patients with relapse were pre-B ALL, one patient was diagnosed with T ALL. Of those patients, two were in the SR group, six were in the IR group, and two were in the HR group. Then, we applied the BFM protocol to those patients with a relapse. Bone marrow transplantation was performed in two patients following remission. One of them, who underwent a fully compatible allogeneic transplant from their sibling, had been followed up for 14 months without any complaints. Yet, the other receiving haploidentical hematopoietic stem cells from their father died. Other six patients with a relapse died from progressive disease.

The serum levels of sCD40 and sCD40 ligand at diagnosis were 22.41±9.91 ng/ml and 15.17±5.49 ng/ml, respectively, whereas these levels at remission were 0.22±0.38 ng/ml and 1.04±0.51 ng/ml, respectively. The changes in the sCD40 and sCD40 ligand levels were statistically significant (p=0.008 and p<0.001, respectively) (Table 3).

We referred to ROC analysis to be able to calculate the cut-off value of sCD40 in determining the early response to treatment (8th day of induction). The findings yielded 28.15 ng/dL as the “cut-value” of sCD40 with a sensitivity of 79.5% and a specificity of 80% (Figure 1). Yet, considering the cut-off value, we could not find significant differences between the risk groups by their sCD40 levels (p=0.085).

Besides, the sCD40 level was not significantly correlated with relapse and survival. Notwithstanding no impact of the sCD40 at diagnosis on relapse and final prognosis, we noted its relationship with early response to treatment.

At the end of the 60-month follow-up, 84.1% (37/44) of our patients were still alive. Accordingly, the overall survival (OS) rate was 82.7±0.60%, and the overall survival time was found to be 51.30 months (46.63-55.98 months) (Figure 2). While the OS rate of the patients younger than six years was detected to be 80.9±8.6%, it was 77.3±8.9% in those older than six years. The OS rate was calculated to be 81.8% within the ten-year follow-up (Figure 3).

Sixty-month EFS was found to be 63.1±15%. In this study, we considered EFS time from diagnosis to relapse or from diagnosis to death due to disease. Then, the EFS time was 49.4 months (43.95-54.84 months) (Figure 4). While the EFS rate of patients younger than six years was 89.8±6.9%, it was found to be 76.0±9.5% among those older than 6 years. The ten-year EFS rate was 61.4% (Figure 5).
DISCUSSION

Thanks to multiple chemotherapies, 80-90% of patients can now be completely cured from ALL, the most prevalent malignant disease in childhood (1, 2). The last 50 years have witnessed significant improvements in survival rates thanks to randomized controlled clinical research, intensive chemotherapy combinations, CNS prophylaxis, and adjusting treatment intensity by risk groups (14,15). Jan Stary et al. previously explored the 5-year outcomes of the ALL-IC BFM 2002 protocol in 15 countries. The findings yielded that the 5-year EFS and OS rates among 5,060 patients were 74% and 82%, respectively. These rates were recorded as 81% and 90% in the SR group (n=1,564), 75% and 83% in the IR group (n=2,650), and 55% and 62% in the HR group (n=846), respectively (13). The previous research also reported various factors affecting the prognosis in ALL patients. The most highlighted ones among these factors are the patient’s age, sex, leukocyte count at diagnosis, and steroid response (1, 2, 16, 17).

We divided the patients into two groups by their sCD40 levels based on the cut-off value yielded by the ROC analysis and explored the OS rate between the groups. Accordingly, we could not conclude that the sCD40 level significantly affected the OS rate. While the OS rate was found to be 81.7±0.65%, the OS time was 51.30 months (45.70-56.90 months) in the group with sCD40 levels less than 28.15 ng/dL. In the other group, these parameters were found to be 84.6±0.10% and 51.23 months (46.63-55.98 months), respectively (Figure 6). When it comes to EFS, despite not being significant (p=0.18), the effect of the sCD40 level on the EFS rate was found to be clinically significant. In the group with sCD40 levels less than 28.15 ng/dL, we found the EFS rate to be 81.6±0.59 % and the EFS time to be 50.85 months (44.95-56.75 months). In the other group, these values were concluded to be 63.1±15.0% and 46.12 months (34.99-57.33 months), respectively (Figure 7).
Our findings showed that the 5-year EFS rate was 89.8±6.9% in our patients aged 1-6 years, while it was found to be 76.0±9.5 among those older than six years. The difference was statistically insignificant, despite being clinically significant (p=0.443). In their multicenter BFM study published in 2008, Mörricke et al. (18) found that 57.9% of 2,169 patients were aged 1-6 years. In the same study, the authors emphasized the effect of age on survival and reported the 6-year EFS rates to be 84.3±1% in those aged 1-6 years and 80.3±1.9% in those the 6-10 years. Accordingly, our findings overlap the survival rates reported in the mentioned research.

Although the chance of treatment success in ALL has reached up to 90%, there is still a paucity of knowledge on the pathogenesis of the disease. The present study explored sCD40 and sCD40L in childhood ALL cases for the first time in the medical literature and concluded their levels to be high at diagnosis. Unlike adult-oriented research, despite a significant link between sCD40 and leucocyte count, absolute neutrophil count, LDH, and ESR, we could not conclude its direct effect on prognosis.

We divided the patients into two by the cut-off sCD40 yielded by the ROC analysis and explored the effect of sCD40 on the OS rate. Our findings showed that sCD40 did not have a significant effect on the OS rate. Yet, we found that its effect on the EFS rate was clinically significant despite being statistically insignificant (p=0.18).

It is well-known that unregulated CD40L production in atherosclerosis and auto-immune diseases continuously stimulates the CD40 pathway. In this respect, it was previously concluded that this pathway plays a role in the pathogenesis of cancer since most malignant hematopoietic diseases and about 75% of epithelial cancers express CD40 (9). In research with patients with non-Hodgkin lymphoma, chronic lymphocytic leukemia (CLL), and Burkitt lymphoma, it was documented that malignant cells autocrinely produce CD40L and secrete it into their microenvironment in order for them to proliferate and survive.

It was demonstrated that the CD40/CD40L interaction, particularly the NF-kB pathway, is blamed for the autocrine proliferation and survival of malignant cells. A similar scenario also applies to melanoma, kidney, and breast cancers that are known to express CD40 and CD40L simultaneously (19, 20). The presence of CD40L in melanoma cells was found to be linked with a more aggressive phenotype and shorter disease-free survival compared to melanoma types not expressing CD40L (19). Accordingly, continuous CD40L stimulation to CD40+ immortalized epithelial cells promotes the proliferation, motility, and invasion properties of these cells, leading to oncogenicity. Similarly, the mitigation capacity of human MM cells was found to be enhanced via the NF-kB pathway following stimulation with CD40 (21). In mouse cancer models, it was reported that angiogenesis was promoted via endothelial cells as a result of chronic stimulation of CD40L to the tumor microenvironment, which indirectly contributed to tumor growth (22). In addition, the CD40/CD40L interaction in neutrophils leads to oxidative burst, resulting in the secretion of free oxygen radicals and MMP, which plays a role in the transformation and metastatic spread (23).

Despite the positive role of the CD40 pathway in cancer pathogenesis at first glance, the role of CD40 in cancer is not that simple. The frequent occurrence of lymphomas and cancers in patients with hyper immunoglobulin M syndrome may indicate that the effect of CD40L on malignant cells is more complex than is understood. Loskog and Eliopoulos (9) point out such a dilemma in their review titled “The Janus Faces of CD40 in Cancer.” In line with this idea, it was reported that tumor cells suppress the CD40 pathway by various mechanisms to be able to survive. It was found that CD4+ cells obtained from CLL patients bear an insufficient expression of CD40L on their surface during in vitro activation and that leukemic cells reduce the expression of CD40L in other allogeneic T cells (24). It was also determined that leukemia cells escape immune surveillance by reducing the expression of CD40L in other T lymphocytes.

A study on adults concluded high levels of sCD40, which inhibits CD40L, at diagnosis among patients with acute myeloblastic leukemia (AML), myelodysplastic syndrome (MDS), chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MHL), and multiple myeloma (MM) and reported that AML and MM patients with elevated sCD40 levels had a poor prognosis (12).

In our study, the high levels of sCD40 at diagnosis may similarly indicate that ALL cells escape from immune surveillance. However, unlike adult patients, the reason why we could not clearly document its effect on prognosis may be due to the small number of patients.

The relationship between the prognosis of leukemia patients and sCD40 is still blurred. Hock et al. (12) suggested that tumors with high sCD40 level are indicative of a more aggressive and chemotherapy-resistant course, independent of other prognostic markers. A study on non-Hodgkin lymphoma with adult patients yielded that single nucleotide polymorphisms in the TNFRSF5 and TNFSF5 genes, which mediate CD40 and CD40L synthesis, cause reduced CD40 expression in B cells. In another study with 1,776 patients with non-Hodgkin lymphoma and 2,482 controls reported an increased risk of non-Hodgkin lymphoma in the case of...
mutations in the TNFRSF5 gene (25). Similarly, Brouwer et al. (26) found a relationship between CD40 (mCD40) in the cell membrane and poor prognosis in patients with AML.

The CD40/CD40L interaction is also effective in the formation of hematopoietic cancers. Adults with leukemia often have high levels of sCD40L, and up to 90% of B-cell neoplasms express CD40 (27). Similarly, CD40L is expressed in a significant portion of T-cell lymphoma cases. The interaction of T-cell neoplasms with CD40+ antigen-presenting cells (APC) leads to cancer cell proliferation. However, the interaction of CLL cells with CD40 initiates the antitumor effect (27). Therefore, CD40L is not only associated with the proliferation and survival of tumor cells but also in the immune response with robust antitumor impacts. Such a contrast is often explained by differences in the activated state of the targeted cells. Blair et al. (28) showed that interaction with CD40L leads resting CD4+ T cells to proliferate but cytokine-activated CD4+ T cells to go into apoptosis. The contrasting effect of the CD40 system on regular and malignant cells reveals how complex the phenomenon is.

Similarly, in their study with B-cell CLL patients, Luqman et al. reported that CD40 expression in blasts stimulated proliferation, rescued cells from apoptosis spontaneously or as a result of chemotherapy, and increased the secretion of cytokines such as IL-6, IL-10, IL-8, TNF-α, and GM-CSF which enable CD40 to survive, migrate, and interact with the tumor microenvironment (11). It was previously reported that HCD122, a monoclonal antibody produced for CD40, inhibits CD40L-mediated signaling pathways and proliferation and survival of cancer cells and can be used for therapeutic purposes by increasing antibody-mediated cell death. It was also documented that HCD 122, a CD40 antibody, is more effective in the cellular response against cancer cells, despite being expressed in fewer cells than the anti-CD20 rituximab (11). Therefore, it was suggested that HCD 122, which can be used in the treatment of CLL, can lead to effective cancer cell death without impairing cells and their functions (29).

CONCLUSION

Unlike research oriented to adults, the relevant literature offers limited knowledge on pediatric patients. Therefore, we investigated the significance of sCD40 and sCD40L levels in childhood ALL cases. In this study, we measured soluble CD40 and CD40L levels for the first time in childhood ALL patients and concluded that they did not have a direct effect on the prognosis, although being found to be higher at diagnosis compared to remission.

Nevertheless, further research is needed to elucidate the role of CD40-CD40L interaction in tumor pathogenesis and treatment in pediatric ALL patients.

ETHICAL DECLARATIONS

Ethics Committee Approval: The study was carried out with the permission of Ethics Committee of the mentioned faculty of medicine (No: 2010/120 dated 10.08.2010).

Informed Consent: All patients signed the free and informed consent form.

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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