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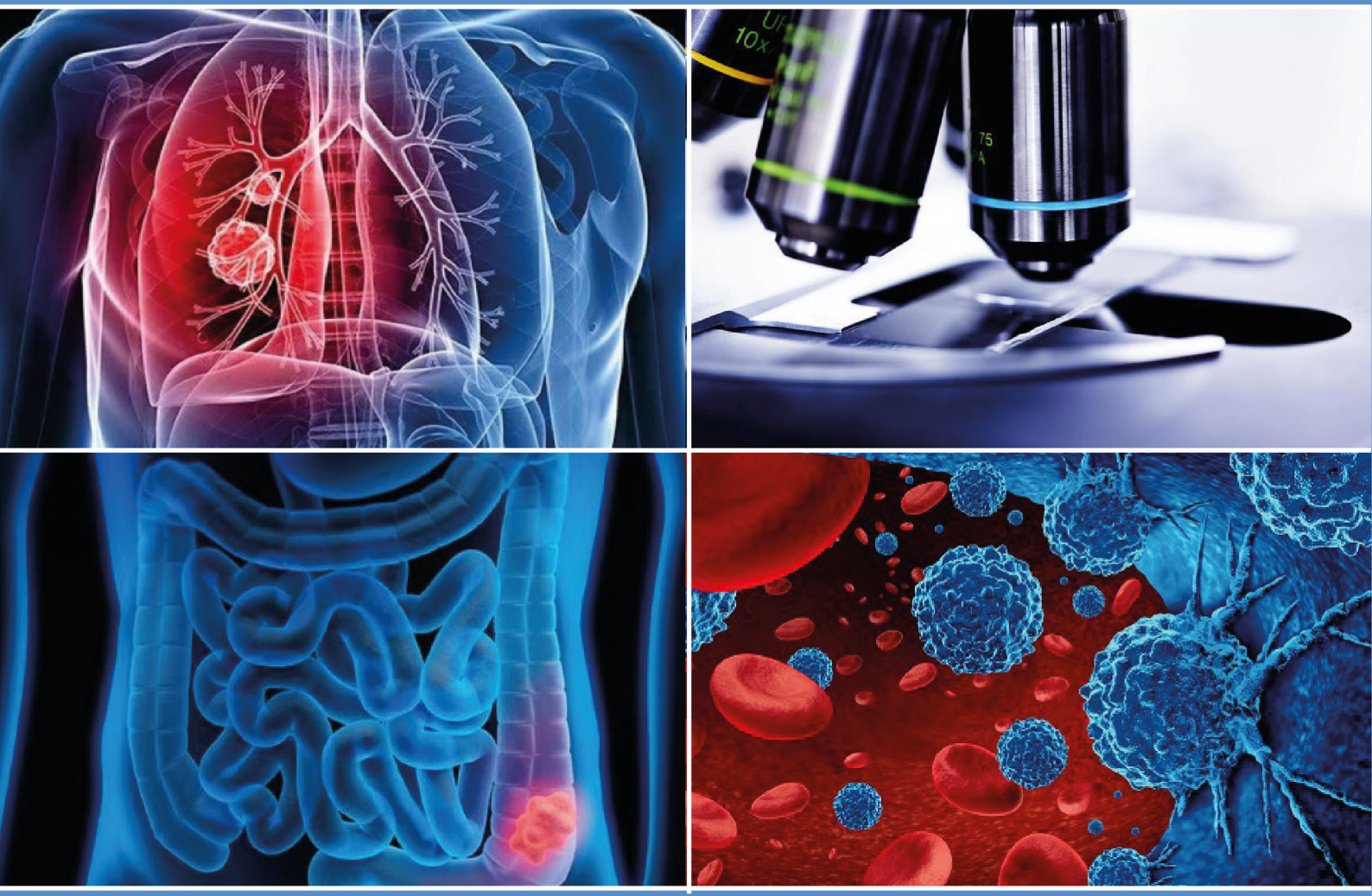

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Original Article

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Determination of the therapeutic effects of apigenin on chronic myeloid leukemia stem cells: a mechanistic approach

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ABSTRACT

Aims: Chronic myeloid leukemia (CML) is a type of leukemia characterized by the Philadelphia (Ph) chromosome encoding the BCR-ABL protein. Significant success has been achieved in the treatment of CML with the development of agents that target this protein and inhibit its activity, but the resistance that develops in patients against these drugs limits absolute success. One of the main reasons for the resistance problem is the ineffectiveness of current therapies for CML stem cells. Apigenin (4',5,7-trihydroxyflavone) is a flavanoid found in various vegetables and fruits and has the effects of suppressing proliferation and inducing apoptosis in different cancer types. In this study, we aimed to determine the therapeutic potential of apigenin on K562 CML stem cells.

Methods: The effects of apigenin on the proliferation of K562 CML stem cells were determined by an XTT cell proliferation assay. Apoptotic effects of apigenin on K562 CML stem cells were determined by changes in mitochondrial membrane potential (MMP), caspase-3 enzyme activity, and the Annexin-V method using flow cytometry.

Results: The proliferation of K562 CML stem cells exposed to increasing doses of apigenin (1-40 nM) for 48 hours decreased dose-dependently, and the IC50 value of Apigenin was calculated at 3 nM. Compared to the control group, an increase in caspase-3 enzyme activity was calculated in stem cells treated with apigenin at the same doses. Disruptions in the mitochondrial membrane potential of apigenin-treated CML stem cells and Annexin-V assay results also showed that apigenin dose-dependently induced apoptosis and caused significant increases in the apoptotic cell population.

Conclusion: It was shown for the first time in this study that apigenin may have therapeutic effects on CML stem cells. It was determined that this effect of apigenin was achieved by triggering caspase-3 enzyme activity and disruptions in the mitochondrial membrane.

Keywords: Apigenin, chronic myeloid leukemia stem cells, apoptosis, cytotoxicity

INTRODUCTION

Chronic myeloid leukemia (CML) is a clonal malignant disease of bone marrow stem cells with a specific chromosomal defect and is characterized by a specific chromosomal abnormality called the Philadelphia (Ph) chromosome. The Ph chromosome is formed by translocation of the BCR and ABL genes located on chromosomes 9 and 22, and the hybrid gene formed as a result of this translocation encodes a protein called BCR/ABL with tyrosine kinase activity that activates signaling pathways that enable uncontrolled growth and division of the cell.[1,2] Although great success has been achieved in the fight against the disease with tyrosine kinase inhibitors targeting BCR/ABL activity, the development of drug resistance is seen as the most important problem encountered in the clinic against these drugs.[3-5] For this reason, many studies are being conducted to overcome the resistance problem that results in the accumulation of primitive cells in the blood and bone marrow. However, in recent years, studies have been developed to develop therapies targeting CML stem cells for the treatment of CML.[6]

Cancer stem cells (CSCs) are known as a subpopulation of small cancer cells with stem cell-like properties and the

ability to produce all cancer cells.[7] Leukemic stem cells (LSCs) play a crucial role in disease incidence, drug resistance, and relapse.[8] LCSCs are regulated by critical surface antigens such as CD34 and CD38 proteins. These proteins are expressed in most cases of leukemia and are therefore used as specific cell markers in both the diagnosis and prognosis of the disease.[9,10] Therefore, targeting these cells will be a beacon of hope in cancer therapy.

Apigenin,(5,7-dihydroxy-2-[4-hydroxypheny]-4H-1-benzopyran-4-one) is a flavone commonly found in onions, grapefruit, oranges, parsley, sand chamomile.[11] Apigenin has an important place in the literature with its potential to be used in cancer prevention and treatment due to its activity in suppressing cell growth in different human cancer cell lines such as leukemia, thyroid, skin, prostate, colon, and breast cancer.[12] This flavone inhibits cancer cell proliferation by triggering cell apoptosis, inducing autophagy, and modulating the cell cycle. Apigenin also reduces cancer cell motility and inhibits cancer cell migration and invasion. Recently, apigenin has been reported to exhibit anti-cancer activities by stimulating an immune response.[13-15] There are studies showing the anti-proliferative effects of apigenin on CML, but no study showing its effect on CML stem cells has been found yet. Targeting cancer stem cells in cancer treatment is promising for cancer patients, as it will reduce the risk of disease recurrence. This study aimed to demonstrate the antiproliferative effect of apigenin on CML stem cells.

METHODS

This study does not require an ethics committee approval.It was approved by Erciyes University Scientific ResearchProject Coordinatorship as a multi-disciplinary research project with project code TCD-2015-5427 All ethical principles were respected in this study. Chronic myeloid cancer stem cells (K562) obtained from the Department of Molecular Biology and Genetics at the İzmir Institute of Technology were used in this study. Flow cytometry results showed that 99.58% of the selected cells were CD38 negative and 94.21% were CD34 positive, indicating that these cells were CML stem cells. These cells were propagated in culture, and the experimental stages of the study were completed. Apigenin, the chemical used in our study, was purchased from Sigma Aldrich (USA). The necessary stock solutions for this chemical were prepared in solvents at the ratios specified by the manufacturer and kept under appropriate storage conditions.

XTT Cell Proliferation Test

An XTT cell proliferation assay was used to determine the cytotoxic effect of apigenin on CML stem cells. These cells were treated with apigenin for 72 hours. For this purpose, CML stem cells were seeded with 10,000 cells in 100 µl of medium in each well of a 96-well plate, and the cells were incubated for 72 hours at 37°C in an incubator containing 5% CO_2 by adding the relevant agents at increasing concentrations in volumes of 100 µl each. At the end of incubation, 20 µl of XTT reagent was added to each well and incubated for 4 hours. Then, the solution formed in 96-well plates was read at 450nm wavelength in a spectrophotometer. A cell proliferation graph was generated according to the spectrophotometric results. The IC50 and IC10 values of all these agents in CML stem cells (the drug dose at which the proliferation of cells is suppressed by 50% and 10% compared to the control group without drug administration) were calculated from the cell growth graph.[16]

Determination of the Apoptotic Effect of Apigenin in CML Stem Cells by Phosphatidylserine Location on the Cell Surface

K-562 cells were seeded with $1x10^6$ cells in 2 ml of medium in each well of a 6-well plate, and cells were treated with increasing concentrations of apigenin and kept in an incubator containing 5% CO_2 at 37°C for 48 and 72 hours. At the end of the incubation period, the cells were centrifuged at 1000 rpm for 10 min, and the cell pellet was homogenized with 1 ml of buffer solution (PBS), and centrifugation was repeated. After centrifugation, 200 µl of Annexin binding solution was added to the cell pellet and homogenized. Then, 2 µl Annexin V and 2 µl propidium iodide were added to this mixture and incubated for 15 minutes at room temperature in the dark. Measurements were then performed using flow cytometry.[17]

Determination of the Effects of Apigenin on Caspase-3 Enzyme Activity in CML Stem Cells

CML stem cells were seeded with $1x10^6$ cells in 2 ml medium in each well of a 6-well plate, and apigenin was added to these cells at increasing concentrations. The cells were kept in an incubator at 37°C with 5% CO_2 for 48 and 72 hours and then centrifuged at 1000 rpm for 10 min. At the end of centrifugation, the cell pellet was homogenized with 50 µl of cell lysis solution. These cells were then centrifuged at 10,000 rpm for 1 min and the supernatant was treated with 200 µl of cell lysis solution. Then, 50 µl of sample, 50 µl of reaction solution, and 5 µl of DEVD-pNA were added to a 96-well plate, two wells for each sample, incubated at 37°C in 5% CO_2 for 2 h, and measured under the 405 nm wavelength in a spectrophotometer. Changes in caspase-3 enzyme activity were determined by proportioning these values measured for each sample to total protein amounts.[17]

Determination of Changes in Mitochondrial Membrane Potential

CML stem cells were seeded with $1x10^6$ cells in 2 ml medium in each well of a 6-well plate, and cells were treated with apigenin alone and incubated at 37°C in an incubator containing 5% CO_2 for 48 hours. When the incubation period was completed, the cells were collected, transferred to separate falcon tubes, and centrifuged at 1000 rpm for 10 min. After centrifugation, the supernatant on the cell pellet was discarded, and the cell pellet was homogenized with 300 µl of medium. Then, 30 µl of JC-1 dye was added to these cells, and the cells were kept in a CO_2 incubator at 37°C for 30 minutes. After this time, the cells were centrifuged at 1000 rpm for 5 min, and then the supernatant was discarded and the cell pellet was homogenized with 200 µl of assay buffer solution. This step was repeated once more, and 320 µl of assay buffer solution was added to the cell pellet to homogenize the cells. Then, 100 µl of each sample was seeded in triplicate in a 96-well plate and measured under 485/560 nm wavelengths in aspectrophotometer.[16,17]

RESULTS

XTT Cell Proliferation Test Results

The proliferation of K562 CML stem cells exposed to increasing doses of apigenin (1-40 nM) for 48 hours decreased dose-dependently, and the IC50 value of apigenin was calculated at 3 nM. The proliferation percentage of K562 CML stem cells treated with 1-, 5-, and 10 nM apigenin decreased by 34%, 80%, and 96%, respectively, compared to the control group (Figure 1).

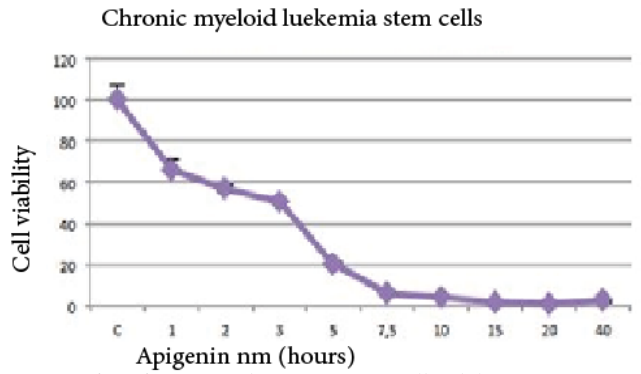

Figure 1. Effect of apigenin administration on cell viability in CML stem cells

Annexin V Test Results

This test is based on the principle that phosphatidylserine, which is found in the inner part of healthy cell membranes, moves to the outer part of the cell with the breakdown of the cell membrane in apoptotic cells. Using flow cytometry and the Annexin-V/Propidium Iodide double staining method, it was possible to determine the location and amount of phosphatidylserine. Annexin-V analysis results of apigenin-treated CML stem cells showed that apigenin dose-dependently induced apoptosis and caused significant increases in the apoptotic cell population (Figure 2).

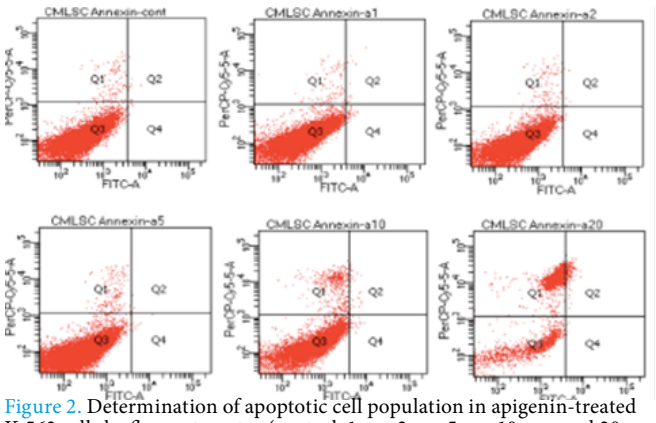

Figure 2. Determination of apoptotic cell population in apigenin-treated K-562 cells by flow cytometry (control, 1nm, 2nm, 5nm, 10 nm, and 20nm, respectively) Caspase-3 Enzyme Activity

Changes in caspase-3 enzyme activity of CML stem cells exposed to increasing doses of apigenin were determined by a caspase-3 colorimetric enzyme kit (BioVision Research Products, USA). This kit is based on the principle that the caspase-3 enzyme recognizes the substrate DEVD sequence and cleaves the DEVD-pNA complex, and as a result of this cleavage, the pNA that emits radiation is detected at a wavelength of 405 nm in a spectrophotometer. Compared to the control group, 8%, 53%, and 82% increases were calculated in caspase-3 enzyme activity of stem cells treated with apigenin at the same doses (Figure 3).

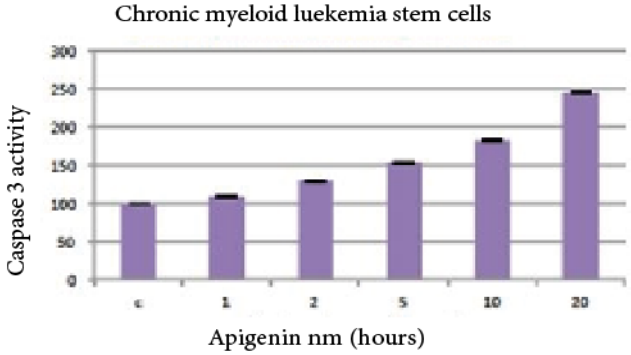

Figure 3. Changes in caspase-3 enzyme activity in K-562 cells treated with increasing doses of apigenin

These results supported that apigenin dose-dependently induced apoptosis and caused significant increases in the apoptotic cell population.

Mitochondrial Membrane Potential Test Results

The JC-1 mitochondrial membrane potential measurement kit (Cayman Chemicals, USA) measured changes in mitochondrial membrane potential in CML stem cells treated with apigenin for 48 hours to determine whether apoptosis had occurred. In light of cell proliferation data, CML stem cells were exposed to increasing doses of apigenin (1nm, 2nm, 5nm, 10 nm, and 20nm) for 48 hours, and changes in mitochondrial membrane degradation were determined. According to the results of the JC-1 assay, an increase of 5nm and above in the mitochondrial membrane potential degradation of CML stem cells exposed to increasing doses for 48 hours was detected. Disruptions in the mitochondrial membrane potential of CML stem cells treated with apigenin showed that apigenin triggered apoptosis in K-562 cells (Figure 4).

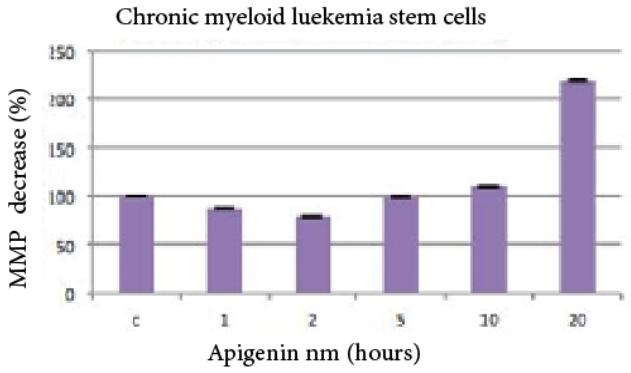

Figure 4. Changes in the mitochondrial membrane potential (MMP)s of K-562 cells with increasing concentrations of apigenin

DISCUSSION

(CML) is a type of leukemia characterized by the Philadelphia (Ph) chromosome encoding the 2010 kDa BCR-ABL (p210) fusion protein.[16] Although significant success has been achieved with the development of tyrosine kinase inhibitors (TKIs) that target the BCR-ABL protein and inhibit its activity and the application of these drugs in CML patients, the resistance that develops against these agents in patients limits the absolute success of CML treatment. One of the main reasons for the

problem of resistance is the lack of efficacy of these agents son CML stem cells.[17-19] apigenin (4',5,7- trihydroxyflavone) is a flavanoid found in different vegetables and fruits and has been shown to suppress cellular growth and trigger apoptosis in different cancer types.[20] In this study, we aimed to determine the therapeutic potential of apigenin on K562 CML stem cells.

Apoptosis is one of the main cell death mechanisms in response to cancer therapy.[21] Apoptosis is controlled by a complex series of interactions between pro-apoptotic proteins (Bax/Bak-like proteins and BH3-single protein) and anti-apoptotic proteins (e.g., Bcl-2, Bcl-xL, Mcl-1) that facilitate apoptosis.[22] Bcl-2 inhibits mitochondrial permeability and subsequent cell death triggered by the pro-apoptotic Bax and Bak.[21,22] Most anti-cancer agents act by triggering the mitochondrial apoptotic pathway involving outer mitochondrial membrane permeability.[19] This process is controlled by pro- and anti-apoptotic members of the Bcl-2 family and causes cytosolic release of mitochondrial intermembrane proteins, including cytochrome c, leading to caspase activation.[19] In CML, treatment options targeting Bcl-2 and the caspase pathway have been shown to be beneficial by suppressing cell proliferation and increasing cell apoptosis.[23,25]

CONCLUSION

In this study, apigenin suppressed cell proliferation in CML stem cells. In addition, the increase in caspase-3 enzyme activity, disruptions in mitochondrial membrane potential, and annexin-V analysis results showed that apigenin dose-dependently triggered apoptosis and caused significant increases in the apoptotic cell population. We believe that these results will guide clinical studies, especially in patients who develop treatment resistance.

ETHICAL DECLARATIONS

Ethics Committee Approval

This study does not require an ethics committee approval. It was approved by Erciyes University Scientific Research Project Coordinatorship as a multi-disciplinary research project with project code TCD-2015-5427.

Informed Consent

Since this study was performed only on the cell line, consent was not obtained.

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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Original Article

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Retrospective evaluation of indications for therapeutic plasmapheresis procedures applied in our center

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ABSTRACT

Aims: Therapeutic plasma exchange (TPE) is a process in which pathological substances (autoantibodies, alloantibodies, immune complexes, lipoproteins, cryoglobulins, etc.) in the patient's plasma are removed from the body along with the patient's plasma and replaced with replacement fluid (allogeneic donor plasma, colloid, crystalloid). It is applied as a complementary treatment method in many diseases that can cause high morbidity and mortality, either in the primary treatment of the disease (main treatment) or as an adjunctive treatment in the main treatment of the disease. Today, TPE is increasingly used in various clinics (toxicology, endocrinology, etc.) for different diseases/causes, especially in hematological, neurological, and nephrological diseases.

Methods: Adult patients who underwent therapeutic plasma exchange in our therapeutic apheresis center between May 2017 and May 2019 were included in the study, and the data were obtained by retrospectively reviewing patient records in the therapeutic apheresis center and our hospital's automation system.

Results: In the two-year period, a total of 1957 TPE procedures were performed on 441 patients. It was determined that 255 of the patients undergoing TPE procedures (57.8%) had indications related to liver diseases (acute liver failure, acute exacerbation of chronic liver failure, and rejection after liver transplantation), while 186 (42.2%) had indications related to non-liver diseases. Among the 186 patients undergoing TPE for non-liver diseases, it was found that 69 (37.1%) were for hematological, 38 (20.4%) for neurological, 32 (17.2%) for renal, and 47 (25.3%) for other diseases.

Conclusion: Due to the large number of liver transplants performed in our Liver Transplant Institute, the majority of TPE indications at our hospital are related to liver diseases. However, the evaluation of TPE indications excluding liver diseases revealed that the indications and frequencies of patients undergoing TPE were consistent with the overall results reported in studies from both our country and abroad.

Keywords: Therapeutic plasma exchange, liver failure, plasmapheresis indications, apheresis types

INTRODUCTION

"Apheresis" is a term derived from Greek, originating from the word "aphairesis," meaning to separate or remove by force. The term was initially used by Rowntree, Turner, and Abel to describe the manual separation of plasma and cellular elements from whole blood using centrifugation methods with heparin.[1] Therapeutic plasma exchange (TPE) is a procedure involving the removal of pathological substances (autoantibodies, alloantibodies, immune complexes, lipoproteins, cryoglobulins, etc.) from the patient's plasma along with the replacement of the removed plasma with replacement fluid (allogeneic donor plasma, colloid, crystalloid).[2] When a substance needs to be removed from the body, and it is (I) too large to be adequately eliminated by treatment techniques such as hemofiltration/hemodialysis (with a molecular weight greater than 15.000 D), (II) has a prolonged half-life exceeding endogenous clearance, and (III) is acutely toxic and/or resistant to conventional treatments, TPE becomes a rational treatment option if at least one of these conditions is present. TPE is utilized in various diseases across different departments, including hematology, nephrology, neurology, rheumatology, and intensive care units, often proving to be life-saving.[3] Primary indications for TPE include diseases such as thrombotic thrombocytopenic purpura, ABO-incompatible kidney transplants, hyperviscosity syndromes, Guillain-Barre Syndrome, and Myasthenia Gravis.[4] The indications for TPE are increasing day by day, and the treatment method is continually evolving.[5] The exact frequency of Therapeutic Plasma Exchange (TPD) application worldwide is not fully known.[6]

In this study, TPE procedures conducted by the Therapeutic Apheresis Center affiliated with our Hematology Clinic were retrospectively examined, and the frequencies of indications for the performed procedures were investigated.

METHODS

The study was conducted at İnönü University, Turgut Özal Medical Center, Hematology Department's Therapeutic Apheresis Center. Patients who underwent therapeutic plasma exchange between May 2017 and May 2019 at our therapeutic apheresis center were included in the study. During this period, a total of 441 patients underwent therapeutic plasma exchange at our center. The therapeutic apheresis indications of the 441 patients who underwent a total of 1957 plasma exchange procedures were retrospectively examined in this study. Adult patients aged 18 and above who underwent plasma exchange at our therapeutic apheresis center were included in the study, while pediatric patients were excluded. Patient information included in the study was obtained by retrospectively reviewing patient records in the therapeutic apheresis center and our hospital's automation system.

For plasma exchange, central catheters were inserted in all patients. After the request for the procedure was made, plasma exchange was performed by calculating 1-1.5 times the plasma volume according to the patient's weight. Plasma exchange procedures were carried out using two different devices: Optia and Comtec, which operate with the centrifuge method.

The study received ethics committee approval from İnönü University Non-interventional Clinical Researches Ethics Committee (Date: 10.12.2019, Decision No: 2019/415). The analyses were conducted in accordance with the principles of the Declaration of Helsinki.

Statistical analysis of the research data was performed using SPSS for Windows Version 22.0 software. Descriptive statistical criteria were used for qualitative and quantitative variables. Qualitative variable data were presented as number (n) and percentage (%), while quantitative variable data were presented as mean±standard deviation.

RESULTS

The plasma exchange procedures performed by our Therapeutic Apheresis Center over approximately a 2-year period were retrospectively evaluated. A total of 441 patients were included in the study, consisting of 232 (52.6%) male and 209 (47.4%) female patients. The average age of the patients was 46.6±16.3 years (range: 18-86 years). The average age of females was found to be 44.9±16.6, while males had an average age of 48.2±16.

The clinics/departments where therapeutic apheresis procedures were performed are shown in Figure, with a breakdown of 879 (45%) procedures in the Liver Transplant Institute, 444 (22.7%) procedures in the Nephrology Department, 153 (7.8%) procedures in the Gastroenterology Department, 134 (6.8%) procedures in the Internal Intensive Care Unit, 132 (6.7%) procedures in the Neurology Department, 87 (4.4%) procedures in Hematology, 52 (2.7%) procedures in the General Surgery Service and Intensive Care Unit, 43 (2.2%) procedures in the Reanimation Intensive Care Unit, 11 (0.6%) procedures in the Chest Diseases Department, 11 (0.6%) procedures in the Rheumatology Department, 5 (0.3%) procedures in the Infectious Diseases Department, 3 (0.2%) procedures in the Medical Oncology Service, 1 (0.1%) procedure in the General Internal Medicine Service, 1 (0.1%) procedure in the Orthopedics Service, and 1 (0.1%) procedure in the Brain Surgery Intensive Care Unit.

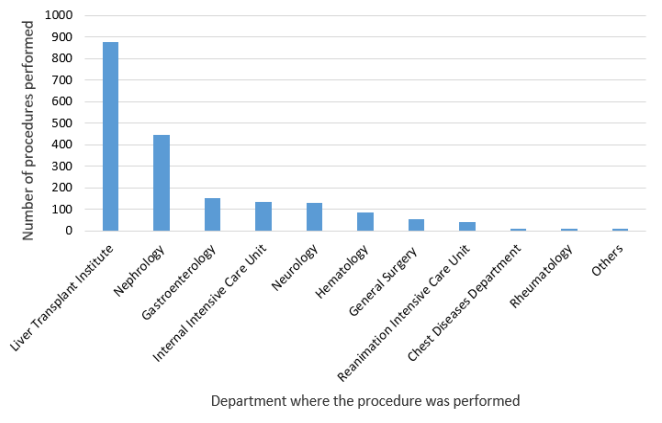

Figure. Departments/clinics where therapeutic apheresis procedures were performed and the number of procedures

Upon classification according to the diagnoses of the 441 patients included in the evaluation, the following results were obtained (Table). Plasma exchange procedures were performed on 228 patients (51.7%) due to conditions such as acute or chronic liver failure developed based on various etiologies such as viral hepatitis, toxic hepatitis. 29 patients (6.5%) underwent plasma exchange with a diagnosis of ANCA-positive vasculitis, including 9 cases of Wegener granulomatosis. Rejection after liver transplantation was observed in 27 patients (6.1%). 22 patients (5%) were diagnosed with rejection after renal transplantation. Plasma exchange procedures were conducted on 18 patients (4.1%) diagnosed with atypical HUS (atypical hemolytic uremic syndrome). HELLP syndrome (hemolysis, elevated liver enzymes, low platelet) led to plasma exchange in 16 patients (3.6%). Multiple sclerosis was diagnosed in 14 patients (3.2%). Thrombotic thrombocytopenic purpura (TTP) was identified in 10 patients (2.3%). Plasma exchange was performed on 10 patients (2.3%) diagnosed with multiple myeloma. Guillain-Barre Syndrome was diagnosed in 9 patients (2.1%). Plasma exchange was applied to 8 patients (1.8%) with a diagnosis of SLE (Systemic Lupus Erythematosus). GVHD (Graft versus Host Disease) was observed in 7 patients (1.6%). Myasthenia Gravis was diagnosed in 6 patients (1.4%). Plasma exchange was performed on 4 patients (0.9%) diagnosed with transverse myelitis. DIC (Disseminated Intravascular Coagulation) was identified in 4 patients (0.9%). Snake bites led to plasma exchange in 4 patients (0.9%). Desensitization before renal transplantation resulted in plasma exchange for 4 patients (0.9%). Good Pasture Syndrome was diagnosed in 3 patients (0.7%). Acute polyneuropathy was observed in 2 patients (0.5%). Plasma exchange was conducted for 2 patients (0.5%) diagnosed with RPGN (Rapidly Progressive Glomerulonephritis). ABY (Acute Kidney Injury) was identified in 2 patients (0.5%). Immune thrombocytopenia led to plasma exchange in 2 patients (0.5%). HSP (Henoch-Schönlein Purpura) was diagnosed in 1 patient (0.2%). Plasma exchange was applied for 1 patient (0.2%) diagnosed with

optic perineuritis. Autoimmune encephalitis was observed in 1 patient (0.2%).

Table 1. Distribution of diagnoses in patients undergoing plasma exchange

Diagnoses	Number of Patients (%)
Viral hepatitis, toxic hepatitis, liver failure	228 (51.7%)
ANCA-positive vasculitis	29 (6.5%)
Liver transplant rejection	27 (6.1%)
Renal transplant rejection	22 (5%)
Atypical HUS (hemolytic uremic syndrome)	18 (4.1%)
HELLP syndrome	16 (3.6%)
Multiple sclerosis	14 (3.2%)
Thrombotic thrombocytopenic purpura	10 (2.3%)
Multiple myeloma	10 (2.3%)
Guillain-Barre syndrome	9 (2.1%)
Systemic lupus erythematosus	8 (1.8%)
GVHD (Graft versus host disease)	7 (1.6%)
Myasthenia gravis	6 (1.4%)
Transverse myelitis	4 (0.9%)
Disseminated intravascular coagulation (DIC)	4 (0.9%)
Snake bites	4 (0.9%)
Renal transplant desensitization	4 (0.9%)
Good pasture syndrome	3 (0.7%)
Acute polyneuropathy	2 (0.5%)
RPGN (Rapidly progressive glomerulonephritis)	2 (0.5%)
Acute kidney injury (AKI)	2 (0.5%)
Thrombocytopenia	2 (0.5%)
Henoch-Schönlein purpura (HSP)	1 (0.2%)
Optic perineuritis	1 (0.2%)
Autoimmune encephalitis	1 (0.2%)
Inclusion body myositis	1 (0.2%)
Multiple organ failure	1 (0.2%)
MPGN (Membranoproliferative Glomerulonephritis)	1 (0.2%)
Intestinal transplantation	1 (0.2%)
Organophosphate poisoning	1 (0.2%)
Hemophagocytic syndrome	1 (0.2%)
Waldenström macroglobulinemia	1 (0.2%)
TOTAL	441 (100%)

Inclusion body myositis was diagnosed in 1 patient (0.2%). Multiple organ failure was observed in 1 patient (0.2%). MPGN (Membranoproliferative Glomerulonephritis) led to plasma exchange in 1 patient (0.2%). Plasma exchange was conducted for 1 patient (0.2%) after intestinal transplantation. Organophosphate poisoning resulted in plasma exchange for 1 patient (0.2%). Hemophagocytic syndrome was diagnosed in 1 patient (0.2%). Plasma exchange was performed on 1 patient (0.2%) diagnosed with Waldenström Macroglobulinemia.

Additionally, out of the 1957 plasma exchange procedures, fresh frozen plasma (FFP) was used as a replacement fluid in 1940 procedures (99.1%), while albumin was used in only 17 procedures (0.9%). Each patient included in the study underwent at least 1 session of plasma exchange, with a maximum of 109 sessions observed.

DISCUSSION

TPE is a treatment method employed for the removal of pathological substances from patients and their replacement with replacement fluid. The indications for TPE may vary from center to center and over time, depending on factors such as center capacity, the development of new treatments, and the intensity of specific patient groups. When considering Turkiye as an example, literature reviews indicate variability in TPE indications and patient diagnoses among different centers. This variability is attributed to factors such as center capacity, the evolution of new therapies, and the specific patient demographics. For instance, a study conducted in Turkiye reveals that TPE, performed on 96 patients, prominently serves as a treatment indication for hematological disorders, particularly conditions such as TTP.[7] In another study conducted at Ankara University, an examination of 658 TPE procedures reported a significant proportion attributed to diseases such as myasthenia gravis and TTP.[8] A broader study conducted across Turkiye, involving 5077 TPE procedures on 1160 adult patients, demonstrated that sepsis/adult respiratory distress syndrome and multiple organ dysfunction were the most common indications for TPE. Additionally, the study highlighted variations in TPE indications between geriatric and non-geriatric groups, with TTP being particularly prominent in the geriatric group.[9] The study titled "Turkiye Therapeutic Plasma Exchange Experience" encompasses 24,912 TPE procedures conducted by 28 therapeutic apheresis centers in Turkiye between 2007 and 2017. In this retrospective assessment, it was determined that the majority of patients fell into categories I and II according to the ASFA criteria. The top five TPE indications were identified as TTP, ABO-incompatible kidney transplantation, hyperviscosity in monoclonal gammopathies, myasthenia gravis and acute inflammatory demyelinating polyneuropathy/Guillain-Barre syndrome. Neurological diseases constituted 36.7%, hematological diseases 31.04%, renal diseases 25.8%, and rheumatological diseases 6.46% of the reported TPE indications.[10]

Excluding a study involving the geriatric patient population, it is generally observed in reported TPE indications from Turkiye that neurological or hematological diseases take precedence. However, this study highlights that liver diseases (such as liver failure and rejection after liver transplantation) constitute the most frequent TPE indication.

In the literature and the guidelines of the American Society for Apheresis (ASFA), the implementation of Therapeutic Plasma Exchange procedures is recommended for acute liver failures[11,12] Acute liver failure can occur due to viral or non-viral causes such as metabolic disorders and the intake of a hepatotoxic substance. As liver damage increases due to the continuous release of endogenous toxic substances and inflammation, on the other hand, the regeneration of the liver is inhibited. Despite various treatments being applied to protect the liver, mortality can exceed 70%. TPE is among these treatment modalities. It is effective in removing endogenous toxins such as endotoxins, bile

acids and bilirubin from the blood and partially replacing the deficiencies in coagulation factors associated with liver failure, thereby correcting coagulation disorders.[13] High-volume plasma exchange (HVP) is a life-saving therapy for acute liver failure (ALF) patients ineligible for liver transplantation (LT), recommended as a primary alternative treatment by the American Society for Apheresis (ASFA). HVP removes toxins, supplements physiological substances, modulates immune responses, promotes liver regeneration, and improves multiple organ dysfunction.[14] According to the results obtained from a study conducted with 31 patients followed up with a diagnosis of acute on chronic liver failure (ACLF); the potential efficacy of TPE in patients with ACLF, suggesting that while TPE may not be effective as a bridge to recovery, it could improve survival rates in selected patients when used as a bridge to transplantation. The retrospective data imply a potential role for TPE in ACLF treatment but underscore the need for cautious interpretation.[15] In another study evaluates the effect of plasma exchange in patients with ALF and ACLF. A literature review revealed that plasma exchange improves survival in ALF patients, particularly those who did not undergo liver transplantation. In ACLF patients, plasma exchange improved survival at 30 and 90 days in non-transplanted patients, indicating the need for further randomized controlled trials.[16]

The results of approximately a two-year period were examined in this study. It was determined that in 57.8% of the patients included in the study (255 out of 441 patients), TPE indications were attributed to liver diseases (such as liver failure and rejection after liver transplantation). This rate was found to be higher compared to those reported in both national and international studies. The primary reason for this is the significant number of liver transplantations conducted annually at the Liver Transplant Institute within the Turgut Özal Medical Center. In this context, the substantial influx of liver disease patients from various regions, particularly from Malatya and its surroundings, seeking treatment at our center, plays a crucial role.

As previously mentioned, our study identified liver diseases as the most common TPE indication. However, upon retrospective evaluation, we found that TPE procedures were performed in 42.2% of the 441 patients due to non-liver diseases. Among these 186 patients, TPE was conducted for hematological diseases in 37.1% (69 patients), neurological diseases in 20.4% (38 patients), renal diseases in 17.2% (32 patients), and other diseases 25.3% (47 patients). Excluding liver diseases and assessing the TPE indications of our center, we determined that our indications and their proportions within the patient population align with the majority of studies reported nationally and internationally.

CONCLUSION

In the retrospective evaluation of Therapeutic Plasma Exchange procedures conducted in our center over the past two years, we identified liver diseases as the most prevalent indication for TPE. The primary reason for this observation is the high number of liver transplantations performed annually within our Liver Transplant Institute. Moreover, when assessing TPE indications excluding liver diseases, we found that the indications and frequencies of patients undergoing TPE align closely with the results reported in studies conducted both nationally and internationally.

In summary, the indications and frequency of TPE may vary depending on the type of diseases, center capacity, and other factors. Studies indicate that TPE can serve as an effective treatment option across diverse clinical scenarios.

ETHICAL DECLARATIONS

Ethics Committee Approval

The ethics committee approval of the study was obtained from İnönü University Non-interventional Clinical Researches Ethics Committee (Date:10.12.2019, Decision No: 2019/415).

Informed Consent

Because the study was designed retrospectively, no written informed consent form was obtained from patients.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Financial Disclosure

There is no conflict of interest between the authors. The authors indicate no financial support or financial conflict of interest. The authors have indicated they have no financial relationships with any company and no external funding.

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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Original Article

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Evaluation of the relationship between immunohistochemical markers and the prognosis of patients with hepatocellular carcinoma

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ABSTRACT

Aims: This study aimed to evaluate whether prognosis and survival time in patients hepatocellular carcinoma (HCC) were associated with immunohistochemistry results for Heat Shock Protein 70 (HSP70), Glutamine synthetase (GS), Cyclase Associated Protein 2 (CAP2), Enhancer of zeste homolog 2 (EZH2) and B-cell-specific Moloney murine leukemia virus integration site 1 (Bmi-1).

Methods: In this retrospective study, the medical records of 50 HCC cases evaluated at Çukurova University Faculty of Medicine, Department of Pathology between 2007 and 2012 were evaluated. Tissues were stained for the targeted antigens. Immunohistochemical stains were scored for cytoplasmic (HSP70, GS, CAP2) or nuclear (EZH2, Bmi-1) staining patterns under light microscopy.

Results: Twenty-nine (58%) of the HCC cases died and the overall survival time was 30±3 (24-37) months. Survival times were similar in terms of age (p=0.262), sex (p=0.707), cause of disease (p=0.655), tumor size (p=0.191) and degree of differentiation (p=0.280). The overall survival of HCC patients with vascular invasion was shorter (p=0.019). The frequency of EZH2 (p=0.025) and Bmi-1 (p=0.004) +/++ was higher in patients with vascular invasion. No correlation was found between overall survival time and HSP70 positivity (p=0.140) and CAP2 positivity (p=0.278); however, survival time was significantly shorter in HCC cases stained (++/+++) with EZH2 (p=0.034), Bmi-1 (p=0.008) and GS (p=0.018).

Conclusion: The results of this study showed that GS, EZH2, and Bmi-1 predicted prognosis and survival time in patients with HCC, possible due to relationships with vascular invasion. There is a need for more comprehensive, population-based studies on biomarkers that can be used in prognosis monitoring of HCC cases.

Keywords: Hepatocellular carcinoma, HSP70, EZH2, GS, CAP2, Bmi-1

INTRODUCTION

Hepatocellular carcinoma (HCC) remains a growing global health challenge, ranking as the fourth leading cause of cancer-related deaths worldwide.[1] Unfortunately, many HCC cases are diagnosed at an advanced stage, resulting in a nearly equal incidence-fatality ratio as demonstrated by data from 2018 showing that there were 841,000 newly diagnosed cases of HCC, leading to 782,000 HCC-related deaths.[1,2] More than 80% of HCC cases occur in East Asia and sub-Saharan Africa, particularly where access to medical and social care resources is limited. However, the burden of HCC has shifted over time from low-middle sociodemographic index regions to high sociodemographic index regions, reflecting the shift from viral to non-viral causes.[3]

HCC occurs mainly in patients with underlying liver disease and is considered one of the leading causes of death in this population. Several well-established risk factors are associated with HCC, including chronic Hepatitis B virus (HBV) or Hepatitis C virus (HCV) infection, alcohol abuse, non-alcoholic fatty liver disease, and exposure to dietary toxins. All these risk factors are potentially preventable and therefore the significant potential of risk prevention to reduce the global burden of HCC should not be ignored.[4] In addition to the need for surveillance, it is also crucial to understand factors that impact the pathogenesis of the disease or its emergence from precursor conditions, including clinically-assessable factors, systemic/metabolic features, and histological characteristics.

HCC precursor lesions show unique molecular alterations of Alpha fetoprotein (AFP), heat shock protein 70 (HSP70), cyclase associated protein 2 (CAP2), glypican 3, and glutamine synthetase (GS), which have proven useful in the histological diagnosis of early HCC.[5,6] These biomarkers are also reported to be associated with poor prognosis in early or advanced HCC. In addition, tumor markers have been widely used in recent years for appropriate treatment selection or response. Although new therapeutic oncological methods have been discovered, the use of biomarkers other than AFP in HCC surveillance in daily practice is limited.[6] In this context, expanding the use of some reliable biomarkers may be beneficial in terms of early diagnosis, predicting prognosis, contributing to the treatment process and positively affecting survival in HCC cases with high mortality.

The aim of the present study was to evaluate whether the prognosis and survival of HCC cases could be associated with various histological markers, including EZH2, Bmi-1, HSP70, GS and CAP2.

METHODS

This retrospective study was authorized in 2013 by the institution of Çukurova University Faculty of Medicine. However, ethics committee approval was not required at that time. Within the scope of the research, liver section data of 50 cases who underwent liver resection for HCC between 2007 and 2012 were evaluated. Clinical data of the cases were obtained from the detailed medical records of the patients in the hospital. For cases with insufficient clinical information, additional information was obtained by contacting the patient or their relatives.

Immunohistochemical Evaluations

The tissues fixed in 10 percent formaldehyde were blocked after the tissue tracking process and hematoxylin and eosin (HE) stained preparations were obtained from 5 micron serial sections. It was examined under a light microscope and suitable blocks were selected. Histological sections were taken on special polylysine slides for immunohistochemical staining. Strept Avidin-Biotin complex immunoperoxidase method was applied to the sections prepared from paraffin blocks of the cases included in the study group (SensiTek HRP, Anti-Polyvalent RTU; HSP70=SPRING BIOSCIENCE, Rabbit Anti-Human HSP70 polyclonal Antibody, CAP2=BIOSS, Rabbit Polyclonal Antibody, EZH2=SPRING, Rabbit Polyclonal Anti–Human, Bmi-1=BETHYL Laboratories Inc, Rabbit polyclonal, Glutamine Synthetase=NOVUS BIOLOGICALS, Rabbit Polyclonal).

Preparation of Tissues For Streptavidin-Biotin Staining

Sections taken from paraffin blocks with a thickness of 5 microns were kept in the oven at 60°C for 30-45 minutes until the paraffin on them melted. The sections were kept in xylene chalk in the same oven for 10 minutes. The sections taken out of the oven were kept in three separate chalks containing xylol at room temperature, then in three separate chalks containing 95% alcohol for five minutes each, and then washed thoroughly in distilled water and the deparaffinization process was completed. To block endogenous peroxidase activity, it was incubated in a solution of 3% Hydrogen Peroxide (H2O2) in distilled water for five minutes. The stages of painting the sections are as follows:

- Antigen retrieval was performed by turning the slides 3 times for five minutes in appropriate solutions in special microwave-resistant chalks (HSP70: Citrate Buffer, pH=6; CAP2: Citrate Buffer, pH=6; EZH2: EDTA Buffer, pH=8, Bmi-1: Tris-EDTA Buffer, pH=9, GS: Citrate Buffer, pH=6). Then, samples were left to cool at room temperature for 50 minutes.
- The sample was washed in Phosphate Buffer Saline (PBS 0.01M) at pH 7.2-7.4 for 3-5 minutes.
- The tissues were placed horizontally in a humid environment, and primary antibody (diluted as required) was added. Incubation was performed at room temperature for approximately 90 minutes (HSP70: 1/150, CAP2: 1/200, EZH2: 1/100, Bmi-1: 1/250, GS: 1/700).
- Wash with PBS (5X).
- Sensi Tek Anti Polyvalent (Scytek) with secondary antibody biotin was added and incubated at room temperature for 15 minutes.
- Washed 5 times with PBS.
- Sensi Tek HRP (ScyTek Laboratories) was added and incubated at room temperature for 20 minutes.
- PBS wash (5X).
- AEC chromogen was added to the sample and incubated for 5-20 minutes, and the tissues were placed in tap water and checked for staining under a microscope.
- The floor was stained with Mayer Hematoxylin for 1-3 minutes and washed with tap water for 3-5 minutes.
- The area around the section was wiped and sealed with water-based sealing agent (Thermo Scientific - Shandon).

Immunohistochemical stains were evaluated according to cytoplasmic or nuclear staining patterns under a light microscope. Nuclear markers Bmi-1 and EZH2 were evaluated with an immunohistochemical score based on the percentage and intensity of staining.7 Staining intensity was scored between 0 and 3 (<5% staining=negative=0, 5–25% staining=sporadic=1, 25–50% staining=focal=2, >50% staining=diffuse=3). Staining intensity was scored between 0 and 3 (No staining=0, Slight staining=1, Moderate staining=2, Severe staining=3). The final immunohistochemical score was calculated with the following formula=(Percentage of Bmi-1 positive tumor area)x(Staining intensity of tumor cells). Accordingly, scoring was made between 0-9 ["-"=(score 0-1), "+"=(score 2-3), "++"=(score 4-6), "+++"=(score >6)]. The same method was used in EZH2. Semiquantitative evaluation was made according to the prevalence and intensity of staining for cytoplasmic markers HSP70, GS and CAP2 (No staining=negative, mild staining=weak positive=+, moderate staining=moderate positive=++, strong staining=strong positive=+++).

Statistical Analysis

All analyses were performed on IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp., Armonk, NY, USA). Shapiro-Wilk test was used to determine whether variables

are normally distributed. Data are given as mean±standard deviation or median (1st quartile - 3rd quartile) for continuous variables according to normality of distribution and as frequency (percentage) for categorical variables. Continuous variables were analyzed with the Student's t test or Mann-Whitney U test / Kruskal-Wallis test depending on normality of distribution and group count. Categorical variables were analyzed with the chi-square test. The statistical significance value was accepted as $p<0.05$.

RESULTS

The research group consisted of 41 (82%) males and 9 (18%) females, with a mean age of 59.1±14.8 (range: 18-94) years. During the examined time period, 29 (58%) patients died. Cause of disease was HBV in the majority of HCC patients (52.0%). The majority of lesions were 4–9 cm in size (60%), 44% of the patients had vascular invasion, and 48% of lesions were well differentiated according to the Edmondson-Steiner grading system. There were no cases with early HCC diagnosis, or were found to have dysplastic focus or dysplastic nodule (Table 1).

Table 1. Distribution of the study group according to descriptive characteristics

	n (%)
Sex	
Male	41 (82)
Female	9 (18)
Cause of disease	
HBV	26 (52)
HCV	10 (20)
HBV+HCV	5 (10)
Unknown cause	9 (18)
Tumor size, cm	
≤3 cm	10 (20)
4-9 cm	30 (60)
≥10 cm	10 (20)
Vascular invasion	
Present	22 (44)
Absent	28 (56)
Degree of differentiation	
Good	24 (48)
Middle	19 (38)
Little	7 (14)
Mortality	
Present	29 (58)
Absent	21 (42)

HBV: Hepatitis B virus, HCV: Hepatitis C virus

The overall survival time of patients was 30±3 months (24-37). Overall survival was not associated with age group (p=0.262), sex (p=0.707), disease cause (p=0.655), tumor size (p=0.191) and differentiation degree (p=0.280). The overall survival of HCC patients without vascular invasion was significantly longer than those with invasion (p=0.019, Table 2).

Table 2. Survival times according to the descriptive characteristics of the study group

	Mortality/n	Survival, month Mean±SD (95% CI)	p
Age groups, year			
≤54 year	9/13	37±6 (26-47)	0.262
55-64	14/18	39±5 (29-50)	
≥65 year	6/19	21±5 (12-30)	
Sex			
Male	24/41	30±4 (22-37)	0.707
Female	5/9	34±7 (20-49)	
Cause of disease			
HBV	16/26	25±6 (13-37)	0.655
HCV	5/10	19±4 (11-27)	
HBV+HCV	3/5	19±6 (8-30)	
Unknown cause	5/9	36±6 (24-49)	
Tumor size, cm			
≤3 cm	8/10	45±5 (36-54)	0.191
4-9 cm	15/30	26±4 (17-34)	
≥10 cm	6/10	19±4 (11-27)	
Vascular invasion			
Present	10/22	16±3 (11-22)	0.019
Absent	19/28	36±4 (28-44)	
Degree of differentiation			
Good	15/24	23±2 (18-28)	0.280
Middle	9/19	24±6 (13-35)	
Little	5/7	37±9 (20-54)	

CI: Confidence interval, HBV: Hepatitis B virus, HCV: Hepatitis C virus

Among the markers, the frequency of strong positivity (+++) was 8% for EZH2, 10% for Bmi-1, 24% for HSP70, 28% for GS, and 34% for CAP2 (Table 3, Figure 1, Figure 2).

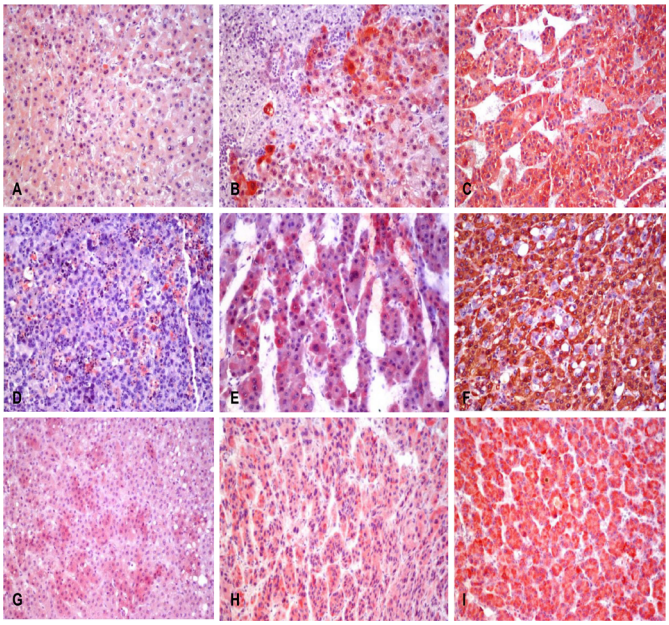

Figure 1. Immunohistochemical evaluations of HSP70, GS and CAP2 [A: HSP70 weakly positive (X200), B: HSP70 moderately positive (X200), C: HSP70 strongly positive, D: GS weakly positive (X200), E: GS moderately positive (X200), F: GS strongly positive, G: CAP2 weakly positive (X200), H: CAP2 moderately positive (X200), I: CAP2 strongly positive (X200)]

Table 3. Distribution of immunohistochemical markers

	n(%)
EZH2	
Negative (-)	29 (58)
+	7 (14)
++	10 (20)
+++	4 (8)
Bmi-1	
Negative	29 (58)
+	3 (6)
++	13 (26)
+++	5 (10)
HSP70	
Negative	4 (8)
+	12 (24)
++	22 (44)
+++	12 (24)
GS	
Negative	16 (32)
+	6 (12)
++	14 (28)
+++	14 (28)
CAP2	
Negative	6 (12)
+	8 (16)
++	19 (38)
+++	17 (34)

EZH2: Enhancer of zeste homolog 2, Bmi-1: B-cell-specific moloney murine leukemia virus integration site 1, HSP70: Heat shock protein 70, GS: Glutamine synthetase, CAP2: Cyclase associated actin cytoskeleton regulatory protein 2

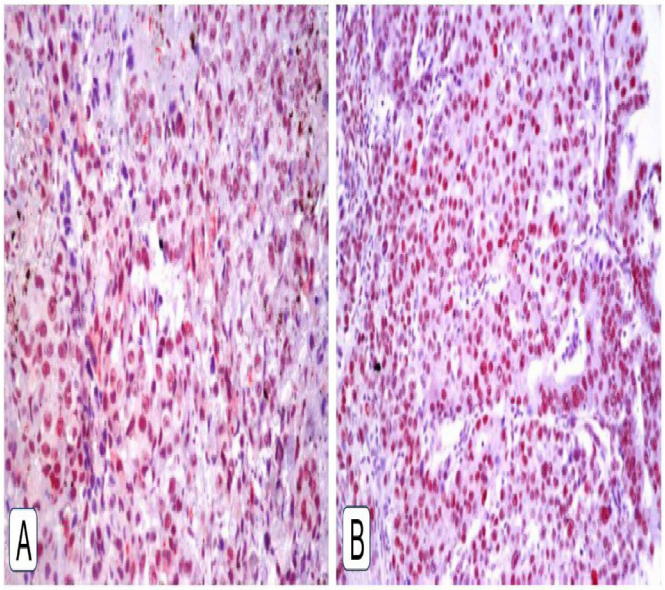

Figure 2. Immunohistochemical evaluations of EZH2 and Bmi-1 [(A: EZH2 nuclear positivity results (X200), B: Bmi-1 nuclear positivity results (X200)]

HSP70 positivity was not associated with age (p=0.122), sex (p=1.000), disease cause (p=0.566), tumor size (p=0.154), vascular invasion (p=0.373) and differentiation degree (p=0.570, Table 4). GS positivity was not associated with age (p=0.375), sex (p=0.481), disease cause (p=0.231), tumor size (p=0.897), vascular invasion (p=0.158) and differentiation degree. (p=0.610, Table 4). CAP2 positivity was not associated with age group (p=0.137), sex (p=1.000), disease cause (p=0.726), tumor size (p=0.589), vascular invasion (p=0.215) and differentiation degree (p=0.892.

Table 4. Distribution of cytoplasmic immunohistochemical markers HSP70, GS and CAP2 positivity according to descriptive characteristics

	HSP70		GS		CAP2	
	-/+ n (%)	++/+++ n (%)	-/+ n (%)	++/++n (%)	-/+ n (%)	++/+++ n (%)
Age groups, year						
≤54 year	3(18.8)	10 (29.4)	7 (31.8)	6 (21.4)	3 (21.4)	10(27.8)
55-64	9(56.3)	9 (26.5)	9 (40.9)	9 (32.1)	8 (57.1)	10 (27.8)
≥65 year	4(25.0)	15 (44.1)	6 (27.3)	13 (46.4)	3 (21.4)	16(44.4)
p	0.122		0.375		0.137	
Sex						
Male	13(81.3)	28 (82.4)	17 (77.3)	24 (85.7)	12(85.7)	29(80.6)
Female	3 (18.8)	6 (17.6)	5 (22.7)	4 (14.3)	2 (14.3)	7 (19.4)
p	1.000		0.481		1.000	
Cause of disease						
HBV	6 (37.5)	20 (58.8)	12 (54.5)	14 (50.0)	6 (42.9)	20(55.6)
HCV	4 (25.0)	6 (17.6)	2 (9.1)	8 (28.6)	4 (28.6)	6 (16.7)
HBV+HCV	2 (12.5)	3 (8.8)	2 (9.1)	3 (10.7)	7 (7.1)	4 (11.1)
Unknown cause	4 (25.0)	5 (14.7)	6 (27.3)	3 (10.7)	3 (21.4)	6 (16.7)
p	0.566		0.231		0.726	
Tumor size, cm						
≤3 cm	5 (31.3)	5 (14.7)	5 (22.7)	5 (17.9)	4 (28.6)	6 (16.7)
4-9 cm	10(62.5)	20 (58.8)	13 (59.1)	17 (60.7)	7 (50.0	23(63.9)
≥10 cm	1 (6.3)	9 (26.5)	4 (18.2)	6 (21.4)	3 (21.4)	7 (19.4)
p	0.154		0.897		0.589	
Vascular invasion						
Present	6 (37.5)	16 (47.1)	7 (31.8)	15 (53.6)	4 (28.6)	18 (50.0)
Absent	10(62.5)	18 (52.9)	15 (68.2)	13 (46.4)	10 (71.4)	18(50.0)
p	0.373		0.158		0.215	
Degree of differentiation						
Good	6 (37.5)	18 (52.9)	9 (40.9)	15 (53.6)	6 (42.9)	18 (50.0)
Middle	7 (43.8)	12 (35.3)	9 (40.9)	10 (35.7)	6 (42.9)	13 (36.1)
Little	3 (18.8)	4 (11.8)	4 (18.2)	3 (10.7)	2 (14.3)	5 (13.9)
p	0.570		0.610		0.892	

HBV: Hepatitis B virus, HCV: Hepatitis C virus, HSP70: Heat Shock Protein 70, GS: Glutamine synthetase, CAP2: Cyclase Associated Actin Cytoskeleton Regulatory Protein 2

EZH2 positivity was not associated with age (p=0.804), sex (p=1.000), disease cause (p=0.956), tumor size (p=0.081) and differentiation degree (p=0.892). Notably, the frequency of EZH2 ++/+++ staining was higher in patients with vascular invasion (p=0.025, Table 5). Bmi-1 positivity was no associated with age (p=0.288), sex (p=0.459), cause

of disease (p=0.287) and degree of differentiation (p=0.865). However, the frequency of Bmi-1 ++/+++ staining was higher in patients with a tumor size of 4-9 cm compared to other values (p=0.037), and in patients with vascular invasion compared to those without (p=0.004) (Table 5).No significant relationship was found between overall survival time and HSP70 positivity (p=0.140) or CAP2 positivity (p=0.278). Overall survival time was significantly shorter in HCC cases stained (++/+++) with EZH2 (p=0.034), Bmi-1 (p=0.008) and GS (p=0.018) (Table 6).

Table 5. Distribution of nuclear immunohistochemical markers Bmi-1 and EZH2 positivity according to descriptive features

	EZH2		Bmi-1	
	-/+ n (%)	++/+++ n (%)	-/+ n (%)	++/+++ n (%)
Age groups, year				
≤54 year	10 (27.8)	3 (21.4)	6 (18.8)	7 (38.9)
55-64	12 (33.3)	6 (42.9)	13 (40.6)	5 (27.8)
≥65 year	14 (38.9)	5 (35.7)	13 (40.6)	6 (33.3)
p	0.804		0.288	
Sex				
Male	29 (80.6)	12 (85.7)	25 (78.1)	16 (88.9)
Female	7 (19.7)	2 (14.3)	7 (21.9)	2 (11.1)
p	1.000		0.459	
Cause of disease				
HBV	19 (52.8)	7 (50.0)	14 (43.8)	12 (66.7)
HCV	7 (19.4)	3 (21.4)	7 (21.9)	3 (16.7)
HBV+HCV	4 (11.1)	1 (7.1)	3 (9.4)	2 (11.1)
Unknown cause	6 (16.7)	3 (21.4)	8 (25.0)	1 (5.6)
p	0.956		0.287	
Tumor size, cm				
≤3 cm	10 (27.8)	0 (0.0)	8 (25.0)	2 (11.1)
4-9 cm	20 (55.6)	10 (71.4)	15 (46.9)	15 (83.3)
≥10 cm	6 (16.7)	4 (28.6)	9 (28.1)	1 (5.6)
p	0.081		0.037	
Vascular invasion				
Present	12 (33.3)	10 (71.4)	9 (28.1)	13 (72.2)
Absent	24 (66.7)	4 (28.6)	23 (71.9)	5 (27.8)
p	0.025		0.004	
Degree of differentiation				
Good	18 (50.0)	6 (42.9)	16 (50.0)	8 (44.4)
Middle	13 (36.1)	6 (42.9)	12 (37.5)	7 (38.9)
Little	5 (13.9)	2 (14.3)	4 (12.5)	3 (16.7)
p	0.892		0.865	

HBV: Hepatitis B virus, HCV: Hepatitis C virus, EZH2: Enhancer of zeste homolog 2, Bmi-1: B-cell-specific Moloney murine leukemia virus integration site 1

Table 6. Distribution of survival times according to immunohistochemical markers

	Mortality/n	Survival, month Mean±SD (95% CI)	p
EZH2			
Negative/+	23/36	34±4 (26-41)	0.034
++/+++	6/14	13±2 (8-18)	
Bmi-1			
Negative/+	22/32	37±4 (29-44)	0.008
++/+++	7/18	12±2 (7-16)	
HSP70			
Negative/+	10/16	35±5 (26-47)	0.140
++/+++	19/34	21±3 (15-26)	
GS			
Negative/+	15/22	38±4 (29-46)	0.018
++/+++	14/28	15±2 (11-19)	
CAP2			
Negative/+	9/14	36±5 (25-42)	0.278
++/+++	20/36	29±4 (21-37)	

HBV: Hepatitis B virus, HCV: Hepatitis C virus, EZH2: Enhancer of zeste homolog 2, Bmi-1: B-cell-specific Moloney murine leukemia virus integration site 1, HSP70: Heat Shock Protein 70, GS: Glutamine synthetase, CAP2: Cyclase Associated Actin Cytoskeleton Regulatory Protein 2

DISCUSSION

HCC is a serious malignant tumor in the world due to its complex molecular and cellular heterogeneity. Besides, HCC incidence continues to increase.[1,2] Over 200 genes related to HCC proliferation, invasion and metastasis have been reported. However, the specific prognostic biomarkers and therapeutic targets are insufficient.[3] Therefore, the screening of HCC molecular biological markers could improve prognosis and reduce mortality.

In this study, we assessed whether some nuclear and cytoplasmic biomarkers could be associated with survival or various other prognostic features in patients with HCC. Our data showed that survival was shorter in patients with EZH2, GS and Bmi-1 positivity; whereas there were no relationships for HSP70 or CAP2. This impact on survival was likely associated with vascular invasion, which was more common in subjects with EZH2 and Bmi-1 positivity.

Possible curative treatments for HCC are liver transplantation, radiofrequency ablation, and resection. However, the effect of these approaches is limited in advanced HCC cases.[8] Advanced stages of HCC can be treated alone or in combination with chemotherapy, immunotherapy and oncolytic viruses. Despite these efforts, high mortality rates are evidence that current treatment options do not achieve the desired therapeutic goals.[9] In the 2015 results of the Global Burden of Disease Study, it was reported that

HBV was the leading cause of liver cancer, death and DALY cases at the global level, followed by alcohol.[2] Similarly, in the current study, the disease cause in the majority of patients diagnosed with HCC was HBV (52.0%). More efforts on HBV vaccination may be beneficial in reducing the prevalence of HCC.

In the Surveillance Epidemiology, and End Results (SEER) database, young age, female sex, Hispanic ethnicity and being married were the determinants that prolonged disease-related survival. Additionally, disease-related survival was worse in patients with greater tumor size (>5 cm), vascular invasion, and lymph node involvement.10 According to the study of Sakamoto et al.[8] in HCC cases, serosal invasion, preoperative AFP, presence of invasion into hepatic veins, and liver cirrhosis were independent predictors of overall survival in multivariate analyses. Another study reported factors that independently affected five-year survival in HCC cases as tumor size >3 cm, involved lymph nodes >2, metastasis, combination therapy with surgery and chemotherapy and coinfection with HBV and HCV.[11] In the current study, overall survival time did not vary according to age group, sex, cause of disease, tumor size and degree of differentiation. The overall survival of HCC patients without vascular invasion was significantly longer than those without. When evaluating factors affecting survival, differences between studies in the factors included in the analyses, clinicopathological status of the cases, treatment regimens, and access to prevention efforts may explain the diversity of results.

In response to the stressful cancer microenvironment, HCC tumor cells can increase the expression of chaperone proteins for cytoprotective function, such as HSP70, GS, CAP2, EZH2 and Bmi-1, resulting in tumor growth and metastasis.[12] Members of the HSP70 family have important roles in protein folding, prevention of protein aggregation, and transport of proteins across membranes under physiological conditions. In environmental (irradiation, chemotherapy), physiological (cell growth, differentiation) and pathophysiological (infection, malignancy) stress situations, the synthesis of the HSP family increases, while protein synthesis generally decreases. Unlike normal cells, the presence of tumors such as HCC causes overexpression of HSP70.[13] It is reported that HSP70 causes the differentiation of tumor cells by stabilizing Cyclin D1 and suppresses the apoptosis of tumor cells by inhibiting the p53 pathway.[14,15] Similar to numerous studies reporting overexpression of HSP70 in HCC cases[12,13,16,17], our results also suggest that HSP70 can be used in the diagnostic process of HCC cases. In addition to being a useful diagnostic marker for HCC, HSP70 also appears to be a predictor of prognosis. High expression of HSP70 was associated with portal vein invasion, but no relationship could be detected between HSP70 and Edmons grade.[18] Joo et al.[16] reported that HSP70 positivity in HCC tissues was positively correlated with tumor size, portal vein invasion, and tumor stage. It has also been reported that HSP70 shows a close relationship with tumor progression, prognostic factors, and pathological parameters.[17] However, Luk et al.[12], reported that HSP70 was unassociated with any of the pathological features examined in their study. In the current study, HSP70 positivity in patients diagnosed with HCC did not vary according to tumor size and differentiation degree, but HSP70 positivity was more frequent in patients with vascular invasion. There was no relationship between survival time and HSP70 positivity.

Another biomarker useful for early diagnosis and prognostication in HCC patients is GS, which causes β-catenin activation. GS-mediated glutamine synthesis in the liver is an important mechanism for ammonia detoxification. In liver malignancy, GS is highly expressed as a transcriptional target of oncogenic β-catenin. Therefore, there is a strong positive correlation between β-catenin activation and GS expression in HCC patients.[19] However, contrary to the widely recognized pro-tumorogenic role of GS, a recent study also reported that GS has a tumor suppressor role in liver cancer by maintaining nitrogen homeostasis through ammonia detoxification.[20] Another study reported that strong positivity for GS was a sensitive marker for HCC in the presence of cirrhosis, independent of tumor differentiation.[21] In the current study, GS positivity was detected in 68% of patients diagnosed with HCC, but was not associated with age, sex, disease cause, tumor size, vascular invasion and differentiation degree. Similarly, there are also results reporting high frequency of GS positivity (53.7%) in HCC cases, but no relationship with clinicopathological parameters. In the study of Morita et al.[22], it was reported that negative staining of β-catenin/GS was associated with both progression-free survival and prolongation of overall survival. Similarly, in this study, we found GS positivity to be associated with a shortened survival time.

In a study by Fu et al.[23], CAP2 expression (53.3%) was reported to be associated with poor overall survival, disease-free survival, and the possibility of relapse. It has been reported that CAP2 was an independent predictive factor for overall survival in multiple Cox regression analysis. Another study reported that CAP2 was a more sensitive biomarker than AFP for early-stage HCC cases, with implications on clinicopathological parameters.[24] In the present study, CAP2 positivity was present in 88% of HCC samples, but no relationship was found between CAP2 and clinicopathological data indicating HCC prognosis and survival.

Bmi-1 and EZH2, which are thought to have active roles in the oncogenic process because they are limited in normal tissue and overexpressed in tumor tissues, are reported to be promising target antigens for cancer immunotherapy.[25] EZH2 plays a role in the cell cycle, DNA damage repair, cell differentiation, autophagy, apoptosis and immunological modulation. The main function of EZH2 is to catalyze the methylation of histone H3K27Me3, which inhibits the transcription of target genes such as tumor suppressor genes. EZH2 also regulates gene transcription by forming complexes with transcription factors or by directly binding to the promoters of target genes. For these reasons, EZH2 inhibition has become an important target for cancer treatment and potential targeting drugs have been developed.[26] A study to identify novel tumor-associated antigens in patients with primary hepatocellular carcinoma reported serological responses to the polyethylene group (PcG) protein Bmi-1, which is overexpressed in a number of different tumor types. In the same study, it was reported that EZH2-derived peptides

caused more significant interferon-γ release than Bmi-1.25 In the study of Li et al.[27], it was reported that the expression of Bmi-1 was increased in HCC tissues and its expression was positively associated with tumor size, metastasis, venous invasion and TNM stage. Additionally, high Bmi-1 expression has been reported to be an independent prognostic factor for overall survival. In the present study, the frequency of EZH2 and Bmi-1 positivity was 42%, and the positivity of both biomarkers was significantly higher in patients with vascular invasion. Additionally, Bmi-1 positivity was higher in patients with larger tumors. When EZH2 and Bmi-1 positivity increased, overall survival time was significantly shortened.

Although it is awaiting standardization, HCC screening is performed every 6 months with AFP levels and ultrasonography.[28] According to the results of the SEER database, there was a significant improvement in the survival rate of HCC patients from 1988 to 2015, which can be attributed to screening efforts, early diagnosis, therapeutic advances, including HBV vaccination.10 Our results indicate that some of these biomarkers may offer unique potential for HCC assessment. Research results supporting this idea continue to be added to the literature. A recent study reported that the EZH2 inhibitor (EZH2i) GSK126 combination was associated with an increase in the number of upregulated genes in HCC cell lines, long-lasting anti-proliferation effects, and increased nucleosome accessibility.[29] In another previous study, it was reported that Hotair silence activates P16(Ink4a) and P14(ARF) signaling by increasing miR-218 expression and suppressing Bmi-1 expression –thereby suppressing HCC tumor formation.[30] There are also results reporting that shRNA-mediated inhibition of Bmi-1 can reduce the invasiveness of HCC (in vitro).[27]

Limitations

The present study includes a small population from a single center and reports results that may have limited generalizability. This is compounded by the fact that the follow-up period was short, and therefore, prognostic or survival analyses may not apply for the mid or long term. The study results could have been strengthened with long-term assessment of HCC prognosis, metastasis and survival. Although our purpose was to examine relationships with clinical data, it could also be valuable to determine relationships between immunohistochemical results and other laboratory data (for instance, AFP levels). In addition, the lack of an evaluation in terms of regimen and treatment duration differences between the patients may have affected the results. Despite all these limitations, this study is valuable in that it provides comprehensive analyses for several crucial cytoplasmic and nuclear biomarkers in HCC cases.

CONCLUSION

The results of this study showed high ratios of positivity for HSP70, GS, CAP2, EZH2, and Bmi-1, indicating their utility as diagnostic markers for HCC. More importantly, EZH2, GS and Bmi-1 were associated with survival, indicating potential use as predictors for prognosis and overall survival. There is a need for more comprehensive, population-based research on biomarkers that can be used in the diagnosis and prognostication of HCC.

ETHICAL DECLARATIONS

Ethics Committee Approval

Since this thesis research was authorized in 2013 by the institution of Çukurova University, ethics committee approval does not require.

Informed Consent

Written consent was obtained from the patient participating in this study.

Referee Evaluation Process

Externally peer-reviewed.

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Conflict of Interest

The authors declare that they have no conflict of interests regarding content of this article.

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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Original Article

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Does early granulocyte colony-stimulating factor administration in autologous peripheral blood stem cell transplantation shorten the duration of hospitalization in patients with multiple myeloma?

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ABSTRACT

Aims: Autologous peripheral blood stem cell transplantation (PBSCT), performed with high-dose melphalan support following induction therapy is still the gold standard method of treatment for multipl myeloma (MM)patients suitable for transplantation. It was aimed, with this retrospective study, to investigate the effects of early (1 day after PBSCT) and late (5 days after PBSCT) initiation of granulocyte colony-stimulating factor (G-CSF) support following PBSCT on engraftment time, febrile neutropenia, and length of hospital stay (LOS) in MM patients.

Methods: This study included 70 patients with MM, who underwent PBSCT in Erciyes University. Two groups were administered 5µg/kg filgrastim, subcutaneously, either 1 day or 5 days after PBSCT, until neutrophil engraftment was reached.

Results: Both neutrophil and platelet engraftment occurred in significantly shorter times in the early G-CSF group compared to late G-CSF group; the median times to neutrophil engraftment were 10 (8-13) and 11 (7-15) days, respectively, and the median times to platelet engraftment were 11 (10- 16) and 13 (11- 21) days (p=0.001). Also, the median LOS was also significantly shorter in the early G-CSF group compared to late G-CSF group; 14 (10-22) vs 16 (11- 33) days, respectively (p=0.016). No significant difference was found between the groups in terms of frequency of febrile neutropenia.

Conclusion: The initiation of G-CSF support early, following PBSCT in MM patients, accelerated neutrophil and platelet engraftment and shortened the LOS as compared to the initiation of G-CSF support late, with no significant difference in the frequency of febrile neutropenia.

Keywords: Autologous hematopoietic stem cell transplantation, granulocyte colony-stimulating factor, multiple myeloma

INTRODUCTION

Autologous peripheral blood stem cell transplantation (PBSCT), is still the standard method of treatment following high-dose chemotherapy in multiple myeloma (MM) patients eligible for transplant, yet the complications associated with prolonged neutropenia have led transplant centers to seek other treatment methods.[1] Administration of granulocyte colony-stimulating factor (G-CSF) after conditioning chemotherapy and stem cell infusion has been shown to expedite neutrophil recovery, decrease time to neutrophil engraftment, and decrease the risk of febrile neutropenia.[2-4] In parallel, the American Society of Clinical Oncology (ASCO) and The National Comprehensive Cancer Network (NCCN) guidelines recommend that G-CSF support should be initiated 1 to 5 days after the administration of high-dose chemotherapy and continued until an absolute neutrophil count (ANC) of 2-3×10^9/L is reached.[5,6] Nevertheless, the data on the optimal timing to initiate G-CSF support is limited and also contradictory.[2,7-9] Also, there are only a few studies that investigated the effect of the timing of the administration of the G-CSF support after PBSCT specifically in the context of MM patients. In this study, the effects of initiating G-CSF early (day 1) or late (day 5) after PBSCT on the neutrophil and platelet engraftment, development of febrile neutropenia, and duration of hospitalization were compared in MM patients. Additionally, the effect of pre-transplant radiotherapy (RT) history on engraftment times was investigated.

METHODS

The study was carried out with the permission of Erciyes University Faculty of Medicine Medical Ethics Committee (Date: 03.11.2020, Decision No: 2020/563). All procedures were carried out in accordance with the ethical rules and the principles of the Declaration of Helsinki.

This retrospective cohort study conducted at Transplantation Center of Erciyes University included adult (age ≥18 years) MM patients undergoing PBSCT using high-dose melphalan conditioning between November 2015, and July 2020. Demographic and clinical features of the patients, induction treatments administered, and their remission statuses before transplantation and history of pre-transplant RT were recorded. Their responses to the treatment before PBSCT were evaluated according to International Myeloma Working Group (IMWG) criteria.[10]

Cyclophosphamide (CY) and G-CSF was used as the mobilization regimen in 31 patients in the first group and 29 patients in the second group. Accordingly, 2.4 g/m^2 CY was administered to the patients on day +1 via intravenous (IV) infusion for two hours, accompanied by Mesna (2-mercaptoethane sulfonate Na) and adequate hydration for the prevention of haemorrhagic cystitis. As the G-CSF, filgrastim (Neupogen, Amgen) 10 µg/kg/d dose was divided into two and started subcutaneously to be administered on day +5 and continued to be administered until sufficient amount of CD34+ cells were collected. Prior to chemotherapy, granisetron or ondansetron, pheniramine maleate, and dexamethasone were administered as IV infusion, whereas acetazolamide support was administered orally. Also this group was given levofloxacin, acyclovir and fluconazole prophylactically, G-CSF was administered along with plerixafor (Mozobil, Genzyme Corp) support due to lack of mobilization in four patients in the first group and five patients in the second group. These patients were administered the filgrastim 10 µg/kg/d dose was divided into two and started subcutaneously to be administered for at least five days and 0.24 mg/kg plerixafor subcutaneously on the fourth day, as suggested in the literature.[11] Additionally, one patient in the second group was mobilized with G-CSF alone; filgrastim 10 µg/kg/d dose was divided into two and started subcutaneously to be administered on day +1 and continued to be administered until sufficient amount of CD34+ cells were collected. The peripheral complete blood count (CBC) measurement was started on day +8 and continued to be performed every other day. Peripheral blood CD34+ cell count was measured daily when patient's white blood cell count recovered to ≥4.000/µL. When CD34+ cell count was ≥10/µL, apheresis was started. Consequentially, adequate doses of CD34+ cells were collected in all patients using a Spectra Optia Apheresis System (Terumo BCT, Lakewood, Colorado, U.S.). Measurements of peripheral blood CD34+ cell count and CD34+ cell content of the apheresis product were performed by the BD FACSCalibur flow cytometer (Becton–Dickinson, Erembodegem, Belgium). The harvested cells were cryopreserved in 10% dimethyl sulfoxide (DMSO) using a controlled-rate freezer, and then stored in liquid nitrogen.

Approximately 2–3 weeks after the mobilization, all patients received conditioning with 200 mg/m² melphalan (140 mg/m^2 in patients with renal insufficiency or >65 years old) two days before the infusion of autologous stem cells, followed by autologous PBSCT on day 0. While the patients who underwent PBSCT between November 2015 and May 2018 received G-CSF at a dose of 5 µg/kg/day subcutaneously starting on post-transplantation on day +5, the patients who underwent PBSCT between May 2018 and July 2020 received the G-CSF at the same dose starting on post-transplantation on day +1. The patients in both groups continued G-CSF treatment until neutrophil engraftment was reached. Also the patients in both groups were given anti-infective prophylaxis, which included 500 mg levofloxacin taken daily, 500 mg valacyclovir taken twice daily, and 400 mg fluconazole taken daily, in accordance with institutional policy. Post-PBSCT neutrophil and platelet engraftment time, development of neutropenic fever, and duration of hospitalization of the two groups were recorded. Neutrophil engraftment was considered the first of three successive days with an ANC≥0.5 x 10^9/L. Also platelet engraftment was considered the first of three consecutive days with a platelet count ≥20x10^9/L. Additionaly, febrile neutropenia was considered the present the fever was ≥38°C and ANC was <0.5×10^9/L from the day of PBSCT until the day of neutrophil engraftment.

Statistical Analysis

SPSS 22.0 (IBM Statistical Package for Social Sciences for Windows, version 22.0, IBM Corp., Armonk, NY, U.S.) software package was used for statistical analyses. The Kolmogorov-Smirnov test was used to check whether the research data conformed to normal distribution or not. Pearson's chi-squared test was used to analyze the independent qualitative data, whereas the student's t-test was used to analyze the independent quantitative data. The Mann-Whitney U test was used to analyze non-normally distributed parameters. Probability (p) values of <0.05 were deemed to indicate statistical significance.

RESULTS

The baseline characteristics of the patients are presented in Table 1. There were 35 patients in each group. The groups were well balanced in terms of age, sex, paraprotein types, disease stage, induction therapies administered, and pre-transplant disease status. Also the groups did not differ significantly in terms of pre-transplant RT history, mobilization protocol, conditioning regimen and CD34+ cell dose.

No serious side effects were observed in both groups during the mobilization and transplantation process. The median time to neutrophil engraftment was 10 days (interquartile range [IQR], 8-13 days) in the early G-CSF group compared with 11 days (IQR, 10-16 days) in the late G-CSF group (p< 0.001) (Table 2 and Figure 1). Also, the median time to platelet engraftment was 11 days (IQR, 7-15 days) in the early G-CSF group compared with 13 days (IQR, 11-21 days) in the late group (p< 0.001) (Table 2 and Figure 2). Additionally, the duration of post-PBSCT hospitalization was 13 days (IQR, 10-22 days) in

the early G-CSF group compared to 16 days (IQR, 11-25 days) in the late group (p= 0.02) (Table 2 and Figure 3).

Table 1. Patient and transplant characteristics

Variables	Early (n=35)	Late (n=35)	P value
Age,yr, median (IQR)	56 (44-64)	57 (40-65)	0.41
Sex, n (%)			0.32
Male	20 (57.1)	24 (68.6)	
Female	15 (42.9)	11 (31.4)	
Isotype, n (%)			0.91
Ig G	25 (71.4)	23 (65.7)	
Ig A	5 (14.2)	8 (22.8)	
Ig D	1 (2.9)	1 (2.9)	
Nonsecretuar	1 (2.9)	1 (2.9)	
Other	3 (8.6)	2 (5.7)	
R-ISS, n (%)			0.26
I	24 (68.6)	27 (77.1)	
II	5 (14.3)	4 (11.4)	
III	1 (2.8)	1 (2.9)	
Missing	5 (14.3)	3 (8.6)	
Pretransplant therapy, n (%)			0.11
VAD+VCD	19 (54.3)	16 (45.7)	
VCD	13 (37.1)	16 (45.7)	
VCD+RD	3 (8.6)	3 (8.6)	
Pretransplant radiotherapy, n (%)			0.16
Yes	11 (31.4)	6 (17.1)	
No	24 (68.6)	29 (82.9)	
Pretransplantation status, n (%)			0.87
CR+VGPR	22 (62.9)	26 (54.5)	
PR	13 (37.1)	9 (45.5)	
Mobilization, n (%)			0.37
Cyclophosphamide + G-CSF	31 (88.6)	29 (82.8)	
Cyclophosphamide + G-CSF+ plerixafor	4 (11.4)	5 (14.3)	
G-CSF only	0	1 (2.9)	
Conditioning regimen,n (%)			0.55
Melphalan 140 mg/m^2	8 (22.9)	6 (17.1)	
Melphalan 200 mg/m^2	27 (77.1)	29 (82.9)	
CD34+ dose, x 10^6 cells/kg, median (IQR)	4.95 (3.22-10.31)	4.99 (3.30-9.86)	0.63

IQR: Interquartile range, R-ISS: Revised international staging system, VAD: Vincristine adriamycin dexamethasone, VCD: Bortezomib cyclophosphamide dexamethasone, RD: Lenalidomide dexamethasone, CR: Complete recovery, VGPR: Very good partial recovery, PR: Partial recovery, G-CSF: Granulocyte colony-stimulating factor

These results were statistically significant. There was no significant difference between the groups regarding the platelet or erytrocyte transfusion requirement. Febrile neutropenia occurred in 20 patients (57.1%) in the early group and 18 patients (51.4%) in the late group (p=0.81). Those patients were treated with infusion of antimicrobial agents and their febrile neutropenia was resolved successfully. There was no relevant difference in both groups in terms of the frequency of febrile neutropenia, relationship of pre-transplant RT history and engraftmen time.

Table 2. Clinical outcomes

	Early (n=35)	Late (n=35)	P value*
Time to neutrophil engraftment,d, median (IQR)	10 (8-13)	11 (10-16)	<0.001
Time to platelet engraftment,d, median (IQR)	11 (7-15)	13 (11-21)	<0.001
Febrile neutropenia,n (%)	20 (57.1)	18 (51.4)	0.81
Duration of hospitalization post-PBSCT,d, median (IQR)	13 (10-22)	16 (11-25)	0.02

IQR: Interquartile range, PBSCT: Peripheral blood stem cell transplantation, *: Statistically significant

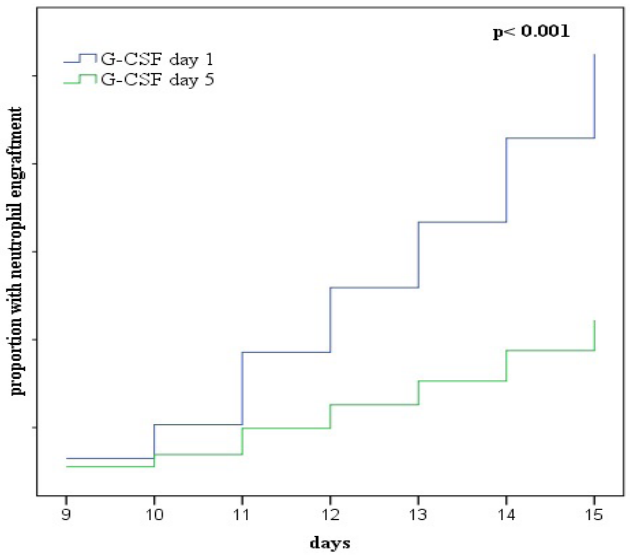

Figure 1. Time to neutrophil engraftment

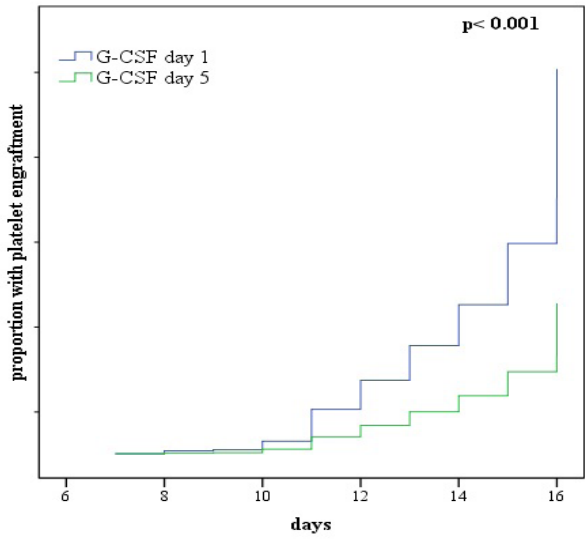

Figure 2. Time to platelet engraftment

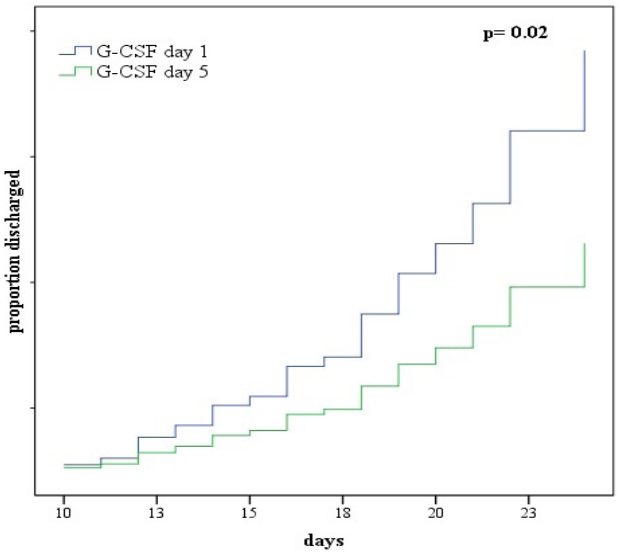

Figure 3. Duration of hospitalization post-transplantation

DISCUSSION

The findings of this study revealed that initiation of G-CSF support early following PBSCT in MM patients accelerated neutrophil and platelet engraftment and shortened the duration of hospitalization. There is no consensus in the literature on the optimum timing to initiate G-CSF support in the post-transplant period. To cite a few examples, in a study by Thompson et al.[7] initiation of G-CSF support on the same day after PBSCT was compared to initiation of G-CSF support five days after PBSCT in the context of various hematological diseases. Consequently, the median time to neutrophil engraftment in the group of patients, who received G-CSF support early, was found as 10 (7-27) days, as compared to 11 (9-15) days in the group of patients, who received G-CSF support late, which indicated a significant difference between the groups in favor of the patient group, who received G-CSF support early (p<0.001). Additionally, in the same study, no significant difference was found between the groups in terms of platelet engraftment times. In a study by Valteau-Couanet et al.[12], patients, who were started on G-CSF support one day after PBSCT, patients who were started on G-CSF support five days after PBSCT, and patients who did not receive G-CSF support, were compared in the context of various hematologic and oncologic malignancies. Consequently, it was determined that the neutrophil engraftment times in the patient groups that received G-CSF support were significantly shorter than those of the patient group that did not receive G-CSF support, whereas there was no difference between the groups in terms of platelet engraftment times. Additionally, in the same study, the neutrophil engraftment times in the patient group that was started on G-CSF support one day after PBSCT, the patient group that was started on G-CSF support five days after PBSCT, and the patient group that did not receive G-CSF support, were found as 9 (4- 40), 10 (5-15), and 13 (7-36) days, respectively. Thus, indicating a significant difference in favor of the patient groups that received G-CSF support (p<0.0001). Furthermore, duration of hospitalization was found to be significantly shorter in the patient groups that received G-CSF support, as compared to the patient group that did not receive G-CSF support. In another study, the difference between the administration of G-CSF support five days after PBSCT empirically and 12 days after PBSCT on patients with an ANC count of <0.5x10^9/L was investigated in terms of engraftment times and duration of hospitalization in patients with MM and lymphoma.[13] Consequently, the neutrophil engraftment times in the patient group that was administered G-CSF support five days after PBSCT and in the patient group that was administered G-CSF support 12 days after PBSCT were found as 12 days and 13 days, respectively. This indicated a significant difference in favor of the patient group that received G-CSF support early (p=0.07). Additionally, in the same study, febrile neutropenia incidences in the patient group that was administered G-CSF support five days after PBSCT and in the patient group that was administered G-CSF support 12 days after PBSCT were reported as 74% and 90%, respectively. This also indicated a significant difference in favor of the patient group that received G-CSF support early (p=0.04). However, no significant difference was found between the groups in terms of platelet engraftment time and duration of hospitalization. The patient group in all these studies consisted of various diseases such as MM and/or lymphoma, solid tumor. In our study, only the data belonging to the MM patients were presented. Few studies were investigated the effect of the timing of the administration of G-CSF support after PBSCT, specifically in the context of MM patients. In one of these studies, Sborov et al.[8] compared the initiation of G-CSF support in MM patients one day, five days, and seven days after PBSCT, and found that the neutrophil engraftment time was shorter and the incidence of neutropenic fever was lower in the group of patients that received G-CSF earlier than others. In the same study, the neutrophil engraftment times in the patient groups that were started on G-CSF support one day, five days, and seven days after PBSCT were found as 12.8 days, 12.3 days, and 11.2 days, respectively. This indicated a significant increase in the patient group that was started on G-CSF support seven days after PBSCT, as compared to the other groups (p<0.001). Additionally, the duration of severe neutropenia was found to be significantly increased in the patient groups that were started on G-CSF support five days and seven days after PBSCT, as compared to the patient group that was started on the G-CSF support one day after PBSCT. Besides that there are two patient groups in our study when there are three patient groups in this study, neutrophil engraftment was occurred in shorter periods in both groups in our study. In addition, a severe neutropenia increase in both groups was mentioned in this study while no such result was found in our study.

In another study, Jackson et al.[14] compared the MM patient group that was administered G-CSF support after PBSCT with the MM patient group, which was not administered G-CSF support after PBSCT, and reported that both engraftment times and duration of hospitalization were significantly less in the patient group that was administered G-CSF support after PBSCT. Additionally, the median times to neutrophil engraftment and the median duration of hospitalization in the said patient groups were found as 12 days and 19 days, and as 15 days and 17 days, respectively, indicating a significant difference between the groups in both categories in favor of the patient group that was administered G-CSF support after PBSCT (p<0.001 and p=0.026). The difference of this study from our study is that no G-CSF support was given to one of the groups, while the other group was given G-CSF in the late period (4-20 days). Also, although the duration of hospitalization with neutrophil engraftment in the group

given G-CSF support was significantly short compared to the group that was not given at all, it was long compared to our early G-CSF group. This result also supports our idea that early G-CSF application is advantageous.

Yet in another study, Cox et al.[15] reported the median neutrophil engraftment times and duration of hospitalization in patient groups that were started on G-CSF support seven days and 14 days after PBSCT as 12 days and 15 days and as 17 days and 19 days, respectively, indicating significant differences between the groups in both categories in favor of the patient group that was administered G-CSF support early ($p<0.0001$ for both cases). G-CSF application days were also different in this study from our study. Moreover, the relationship between the history of pre-transplant RT and engraftment kinetics was not examined in any of these studies. As is seen, there is no research in the literature that our study exactly overlaps.

As for the effect of having a history of pre-transplant RT on the engraftment times, no significant effect was observed in either patient group. This result differs from the relevant results reported in the literature, which indicated that pre-transplant RT significantly affected and delayed platelet engraftment. In those studies, those results may be associated with distorted marrow microenvironment due to RT.[16,17] Data belonging to various cancer patients were shared in those studies.

Limitations

There are some limitations to our study. The first is that patients were not evaluated in terms of total survival and relapse. Because most of the patients who are transplanted in our center come from other provinces and can have their follow-up done in the provinces where they are located after the transplantation. The second might be the cost analysis. The costs of all procedures in the transplantation process of the groups, mobilization and transplant preparation regimes can be affected by various factors. For example, depending on the condition of the patient and the clinic, the mobilization process may be performed by hospitalization or outpatient follow-up. The use of plerixafor in case of failure in mobilization affects the cost. In other words, it may be appropriate to plan a prospective study for a cost analysis based on the duration of post-transplantation hospitalization.

CONCLUSION

It was found that initiating the G-CSF support one day after PBSCT, as compared to five days after PBSCT, significantly shortened the neutrophil and platelet engraftment times as well as duration of hospitalization in MM patients. The results of this study support the transplant centers that reported a positive contribution of early G-CSF support on engraftment and duration of hospitalization.

ETHICAL DECLARATIONS

Ethics Committee Approval

The study was carried out with the permission of Erciyes University Faculty of Medicine Medical Ethics Committee (Date: 03.11.2020, Decision No: 2020/563).

Informed Consent

Because the study was designed retrospectively, no written informed consent form was obtained from patients.

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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Review

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Superior vena cava syndrome

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ABSTRACT

Superior vena cava syndrome is the general name for the symptoms and presentation due to acute obstruction or occlusion of the superior vena cava flow. It usually develops secondary to underlying malignancies and is a life-threatening oncologic emergency. In this review, the current clinical approach to superior vena cava syndrome, including the etiologic considerations, investigations that should be planned, diagnosis, and treatment algorithms, is reviewed.

Keywords: Superior vena cava syndrome, oncology

INTRODUCTION

Superior vena cava syndrome (SVCS) is the name given to the symptoms and general picture that develop due to obstruction or occlusion of blood flow in the superior vena cava, which consists of a thin wall, and it is a condition that may have a mortal course.[1,2] It frequently occurs due to thrombus formation or infiltration of the vessel wall by malignant cells.

ANATOMY AND PHYSIOLOGY

The superior vena cava (SVC) is a very important structure that provides venous drainage of the head, neck, upper extremities, and upper thoracic region and accounts for one-third of the total venous return to the heart. It is structurally thin-walled and valveless, which makes it highly sensitive to compression by surrounding lesions. The SVC is formed by the junction of the right and left brachiocephalic veins at the inferior posterior aspect of the right first costa. The venous structures constituting the SVC are summarized in Figure 1.[3]

Figure 1. Venous structures forming the SVC

The SVC travels along the mid-upper part of the mediastinum and empties into the right atrium of the heart at the level of the third intercostal space. This journey of the SVC is approximately 7 cm, and the vessel width is around 2 cm. The SVC is frequently exposed to obstruction or compression due to the many structures in its neighborhood. These structures include the sternum and trachea, the pulmonary artery, the right bronchus, and surrounding lymph nodes. In addition, infiltration due to malignancies may also lead to obstruction. When obstruction develops in the SVC, alternative routes to the right atrium are formed through collateral vessels due to increased pressure in the surrounding veins. It takes several weeks for collaterals to become evident after obstruction. The most important structures providing these pathways are the azygos vein, hemiazygos vein, internal mammary vein, and lateral thoracic vein. Venous pressure decreases with the effect of collateral vessels. However, this decrease in pressure is transient, and if the underlying cause is not eliminated, the pressure rises again, and the classical symptoms of SVCS are established. Especially in obstructions below the azygos vein, the clinic develops more rapidly and more prominently.[4,5]

Recognizing anatomic variations is becoming increasingly important with the increasing frequency of interventional treatments in recent years. The most common congenital anomaly of the SVC is persistent left SVC, and its frequency in the general population is approximately 0.4%.[6]

ETIOLOGY

While infectious causes (such as tuberculosis and syphilis) were frequently observed in the etiology of SVCS in the past

few years, malignancies are now at the forefront. Malignant causes constitute approximately 70% of the etiology. Among malignant causes, non-small cell lung cancer (NSCLC) ranks first. It has been found that approximately 50% of all malignant causes originate from NSCLC. The second most common cause is small cell lung cancer (SCLC), with a prevalence of approximately 25%. These are followed by lymphoma subtypes. The etiology of SVCS is summarized in Table 1.[2]

Table 1. Etiologic causes in superior vena cava syndrome

Malignant causes (about 70%)	Benign causes (about 30%)
NSCLC SCLC Lymphomas (especially NHL) Thymoma Other mediastinal and metastatic cancers	Mediastinal fibrosis Thrombosis Tuberculosis and fungal infection Vasculitis (often Behcet's syndrome) Radiation-induced fibrosis Aortic aneurysm Sarcoidosis and silicosis

Among benign causes, the increased use of intravenously implanted devices such as pacemakers, port catheters, and implanted defibrillators, especially in the last decade, paves the way for SVCS by bringing thrombotic side effects and increasing its incidence. In clinical studies, it has been observed that 28% of all SVCSs are device-related.[2] The frequency of benign causes of SVCS not related to devices and catheters is decreasing day by day. Patients with SVCS due to benign causes are generally younger and have a longer life expectancy.[7-10]

EPIDEMIOLOGY

The incidence of SVCS is reported to be approximately 15,000 cases per year in the USA, and studies show that the incidence is increasing. In the literature, the incidence of SVCS is between 1/650 and 1/3100. In clinical studies, it has been determined that the incidence has increased, especially in recent years, as a result of the increase in the use of catheters, pacemakers, and defibrillators.[11]

CLINICAL FINDINGS AND DIAGNOSTIC APPROACH

Clinical findings in SVCS present a wide range and vary according to the severity of obstruction, anatomical localization, rate of development, etiologic cause, and performance of the patient. The most common clinical findings include facial and neck edema, neck and chest vein engorgement, watery eyes, and upper extremity edema. These clinical findings and their frequencies are compiled in Table 2.[12]

In patients with SVCS developing due to malignant conditions, a sudden increase in venous pressure may occur due to the rapid occlusion of the SVC. Life-threatening cerebral and laryngeal edema may develop in these patients.[12] Clinical findings are sufficient for the diagnosis of SVCS in many patients. Confirmation of the diagnosis with radiologic imaging is not essential.Although it is important to make a diagnosis in a patient with SVCS clinic, it is also essential to determine the etiology of this condition and the subtype of malignancy, if any.

Table 2. Symptoms and clinical findings in superior vena cava syndrome

Symptoms and Findings		Frequency of Occurrence
Hemodynamic findings	Facial edema	82%
	Edema in the arms	46%
	Fullness in the neck veins	63%
	Fullness in the chest veins	53%
	Facial plethora	**20%**
Respiratory finding*	Symptoms related to vision	2%
	Dyspnea	54%
	Cough	54%
	Hoarseness	17%
	Stridor	4%
	Syncope	10%
Neurological findings	Headache	9%
	Dizziness	6%
	Confusion and stroke	6%

A detailed history and a good physical examination are very important for patients with clinically suspected SVCS. The severity of the clinic is of great importance in terms of the need for urgent treatment. A scoring system evaluating the severity of the clinic in SVCS syndrome has been organized and is presented in Table 3.[13]

Table 3. Clinical severity classification of patients with superior vena cava syndrome

	Severity		Description
0		10%	Radiologic findings, no clinical findings
1	Lightweight	25%	Head and neck edema, cyanosis, plethora present
2	Middle	50%	Accompanied by functional impairment (difficulty swallowing, cough, restriction of neck and eye movements, visual impairment, etc.)
3	Heavy	10%	Mild/moderate brain edema (headache, dizziness, mild laryngeal edema, syncope)
4	Life-threatening	5%	Severe cerebral edema (confusion), severe laryngeal edema (stridor), severe hemodynamic problems (syncope without triggering factor, hypotension, renal failure)
5	Fatal	<1%	Death

Radiologic studies are important to determine the etiologic cause, to determine the secondary interventional diagnostic method, if any, and to determine treatment management rather than diagnosis. For example, a mediastinal mass can be diagnosed with a simple chest radiograph, but the method that will present the content of the tissue and its relationship with surrounding tissues in detail will be computed tomography.

Contrast-enhanced computed tomography (CT) is particularly preferred in these patients. Contrast-enhanced CT is the best method of visualization of the SVC and is also very helpful in determining the site of endovascular intervention for patients for whom intervention is planned.14 Computed tomography, especially in the venous phase, is important in terms of a

better evaluation of the VCS. Contrast venography is of great importance in patients in whom stent placement or operation is planned. This method provides the most detailed and accurate information about the location and degree of obstruction and accompanying thrombosis in the SVCS.[15]

The radiologic classification of SVCS was made by Stanford et al.[16] and is presented in Table 4.

Table 4. Radiological classification of superior vena cava syndrome

Type 1	There is a mild degree of stenosis <90%
Type 2	There is severe stenosis >90%
Type 3	There is complete obstruction but the mammary and epigastric veins do not participate in collateral flow.
Type 4	There is complete obstruction, the mammary and epigastric veins participate in the collateral flow.

Since the treatment of SVCS is targeted at the underlying disease, histopathologic diagnosis before treatment is very important. Currently, the most commonly used method is transbronchial fine needle aspiration biopsy with bronchoscopy, and the diagnosis rate is quite high. Nevertheless, all diagnostic methods should be evaluated, and the most appropriate choice should be made for the patient. In a retrospective study, the methods used in histopathologic diagnosis and their diagnostic rates are shown in Table 5.[17]

Table 5. Histopathologic diagnostic methods and rates of use in superior vena cava syndrome diagnosis

Diagnostic Method	Rates of Use in Diagnosis
Fiberoptic Bronchoscopy	65.7%
Transthoracic biopsy	17.1%
Video thoracoscopy	8.6%
Mediastinoscopy	5.7%
Peripheral lymph node excision	2.9%

TREATMENT APPROACH

Patients diagnosed with SVCS should definitely be followed up in a multidisciplinary manner. In order to evaluate different treatment options, especially oncology, pulmonology, radiology, radiation oncology, and surgery, specialists should follow the patient closely.[18]

There are two main components of treatment for patients diagnosed with SVCS. These can be listed as symptomatic palliation and treatment of the underlying disease. Conservative treatment is applied until the underlying disease is diagnosed. Although there are many opposing views on conservative treatment, the definitive methods are bed rest, bed head elevation, oxygen support, and balanced fluid intake. The first conservative treatment is the elevation of the head of the bed. The aim of elevating the head of the bed is to decrease the hydrostatic pressure in the head and neck and to decrease the clinical findings of SVCS.[19]

There are also methods that are recommended but for which there is no definite data on their usefulness. These can be listed as diuretics and corticosteroids. Diuretics show their effect by decreasing intravascular pressure in venous structures distal to the obstruction. Corticosteroids are especially effective in corticosteroid-sensitive malignancies, but their use is recommended for a limited period.[12]

Clinical severity staging is very important in specific treatment for underlying conditions. In such patients, there are different approaches depending on the stage. This approach is summarized in Figure 2.[17]

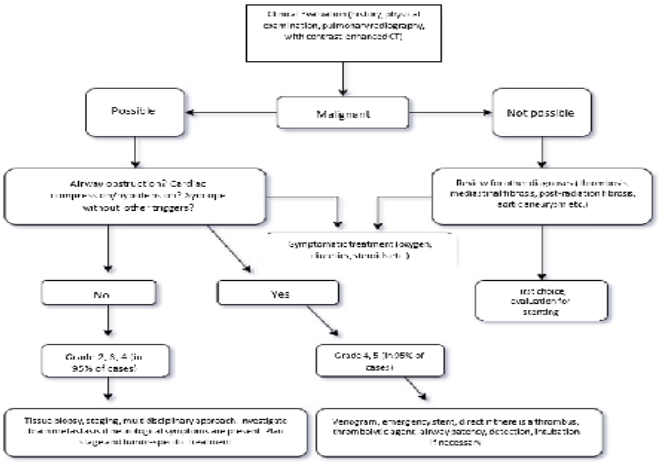

Figure 2. Clinical approach algorithm for a patient with superior vena cava syndrome

Until the recent past, radiotherapy was considered an emergency and first-line treatment for SVCS. However, new studies have looked into other options because radiotherapy has a lower chance of making a histopathologic diagnosis, symptoms can last up to three weeks longer, and the effects are only temporary. With the development of vascular intervention technologies, endovascular stent applications have been shown to improve symptoms faster and more effectively.[20-22]

Among endovascular therapies, especially stenting, has become the standard approach for SVC obstruction for both benign and malignant etiologies in the last 2 years. The benefits of this treatment include a high success rate and a low complication risk. In addition, it does not affect the histopathologic diagnosis and can be used in combination with chemoradiotherapy.[19]

Balloon angioplasty alone is an approach that does not require additional treatment, rapidly restores flow, and provides symptom improvement. However, early restenosis and reocclusion are frequent due to external compression and the fibroelastic structure of the perivascular tissue. Therefore, current guidelines recommend stent placement in most cases of SVC occlusion. Correct stent selection is important in these patients. Stent selection depends on many factors, including the severity of SVC obstruction, length, tortuosity, and resistance to dilatation.[23,24] Accordingly, the most commonly used stents are balloon-expandable stents, self-expanding wall stents, and Gianturco Z-stents.[25]

Unfortunately, surgical options are limited in SVCS. It is considered in cases of severe occlusion and thrombosis of

the collaterals. It is usually a quick and permanent solution. However, its invasiveness causes it to lag behind other options. Chemotherapy/radiotherapy may be preferred due to their high sensitivity, especially in cases caused by SCLC. The commonly used regimen is the platinum-etoposide combination. In NSCLC, radiotherapy is more prominent. There are clinical studies showing that the use of targeted agents is also beneficial. Another important method is endovascular stenting. It can be used alone or in combination with chemoradiotherapy. Clinically, it has been shown to be roughly 90% effective. It should be considered as the first choice, especially in cases requiring urgent intervention.[9,26]

The prognosis of SVCS depends on the underlying cause and treatment. The prognosis is generally poor in patients with cancer. In SVCS caused by thrombosis or mediastinal mass, it is possible to improve symptoms and prognosis with treatment.

CONCLUSION

SVCS is a constellation of clinical signs and symptoms that result from partial or complete obstruction of the SVC. In addition to early diagnosis, significant advances are also needed in the treatment of SVCS. Targeted therapies and immunotherapies can offer patients more effective treatment options with fewer side effects.

SVCS also has psychological and social dimensions, in addition to physical ones. Therefore, psychosocial support and rehabilitation programs should also be developed to help manage symptoms and improve patients' quality of life.

We believe that future research will play a key role in the fight against superior vena cava syndrome (SVCS). The primary objective of this research is to review the current approach to superior vena cava syndrome and to pave the way for future treatment modalities.

ETHICAL DECLARATIONS

Referee Evaluation Process
Externally peer-reviewed.

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

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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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Case Report

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A rare blood transfusion complication: *Bacillus thermoamylovorans* bacteremia and diffuse pustular rash

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ABSTRACT

Bacillus thermoamylovorans is a species of bacillus that causes structural defects in milk by contaminating milk with its heat-resistant spores. We report a case of diffuse pustular rash on the extremities and *bacillus thermoamylovorans* bacteremia in blood culture after erythrocyte suspension replacement. On admission, his acute phase reactants were elevated and he had a generalized rash. After appropriate antibiotherapy, his lesions regressed almost completely. There is no case report of blood transfusion-associated *bacillus thermoamylovorans* bacteremia in the literature. This case is presented to contribute to the literature to show that *Bacillus thermoamylovorans* bacteremia may play a role in the etiology of pustular rash and infectious reactions associated with blood transfusion.

Keywords: *Bacillus thermoamylovorans*, rash, transfusion reaction

INTRODUCTION

Transfusion reactions occur in approximately 1% of all blood transfusions.1 Reactions due to blood transfusion are usually not life-threatening, but although rare, one in 200,000–420,000 units of transfusion reactions result in death.[1] Transfusion reactions are classified as immunologic and non-immunologic. Infectious reactions, which are non-immunologic transfusion reactions, can be caused by bacterial, viral, parasitic and fungal agents. Transfusion-transmitted bacterial infections (TTBI) are much more common than viral and parasitic infections.[2]

In this case report, a rare case of Bacillus thermoamylavorans bacteremia presenting with a diffuse pustular rash after erythrocyte suspension (ES) replacement is presented.

CASE

A 71-year-old male patient with known type 2 diabetes mellitus, benign prostatic hyperplasia and myelodysplastic syndrome, presented to our center with bilateral edema and red-purple non-blanching pustular rashes and bullous lesions on the upper and lower extremities that started 48 hours after red blood cell suspension replacement at an external center. The patient's rash had gradually increased in the last 2 days and spread to all of his arms and legs, he had a fever exceeding 38.0°C during this period.

On physical examination of the patient who presented to us on the fourth day of the rash, diffuse edema was observed in his bilateral hands and feet, as well as purpuras tending to merge on the soles of his feet, dorsum of his feet, and ankles. Purpuric plaques with pustules on them were more intensely present on the upper ankle and dorsum of the hand, decreasing in bruising and intensity towards the proximal. Bullous lesions were also observed on the dorsum of his right hand (Figure 1).

Oral mucosa was normal. There was no history of newly started medication or antibiotics in the patient's history. The patient had no history of raw milk products consumption but drank boiled milk in his daily routine. There was no change in his diet recently. The patient, who retired four years ago and was not actively working, waspreviously engaged in farming.

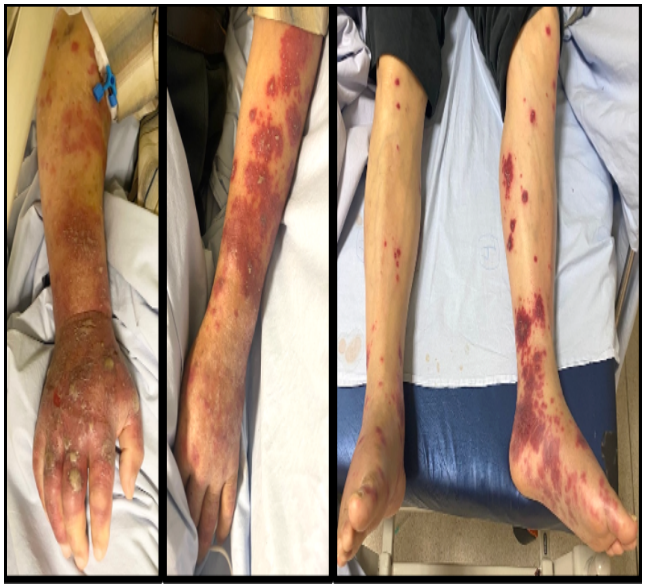

Figure 1. Images of the patient's rash at the time of admission to our center (4 days after erythrocyte suspension replacement, 2 days after the onset of the rash)

The patient was consulted to the dermatology department and skin biopsies were performed with the prediagnoses of pustular vasculitis, Sweet's syndrome, and acute generalized exanthematous pustulosis. The patient had pancytopenia. hemoglobin: 6.0 g/dl (13.5-16.9 g/dL); leukocyte: 3.88x103/µL (3.91-10.9x103/µL) neutrophil: 2.75x103/µL (1.8-6.98x103/µL); platelet: 41x103/µL (166-308 x103/µL), ESR 30 mm/h (0-20 mm/h), CRP; 14.2 mg/dl (0-0.8 mg/dl) and procalcitonin; 0.42 ng/mL (0-0.1 ng/mL) were the results. herpes virus type 1 and type 2 polymerized chain reaction results were negative in the samples taken from the patient's bullae and blood. Rheumatologic markers sent for vasculitis were negative. In the follow-up, intravenous clindamycin 3x600 mg/day and cefazolin 3x2 gr/day were started empirically as the patient's lesions increased, acute phase reactants increased and skin ultrasonography was suggestive of infective pathologies. The patient's skin biopsy result was reported as "crust and bacterial impetiginization findings were observed on the surface of the sections, epidermis was generally normal, dense erythrocyte extravasation on the surface of the dermis, perivascular mild inflammation consisting of lymphocytes was observed. Kappa, lambda, IgG, IgA, IgM and C3 immunofluorescence was negative both in the vessel walls and epidermis. No evidence of vasculitis or drug reaction was detected. There is diffuse erythrocyte extravasation on the surface." There was no bacterial growth in the cultures obtained from skin lesions, but *Bacillus thermoamylovorans* grew in the blood culture. Bacterial growth time was reported as 2 days, 7 hours and 55 minutes. Colony count not specified.

Due to the presence of *Bacillus thermoamylovorans* species producing ßeta lactamase in the literature, cefazolin treatment was stopped and teicoplanin treatment was started. Clindamycin treatment was continued. No growth was observed in cultures obtained from separate vein and repeated blood cultures due to initiation of empirical treatment. The patient, who completed teicoplanin treatment in 14 days and clindamycin treatment in 10 days, showed almost complete regression in acute phase reactants(ESR; 17 mm/h (0-20 mm/h), CRP 3.97 mg/dL (0-0.8 mg/dL) and procalcitonin 0.13 ng/mL (0-0.1 ng/mL)) and skin lesions (Figure 2).

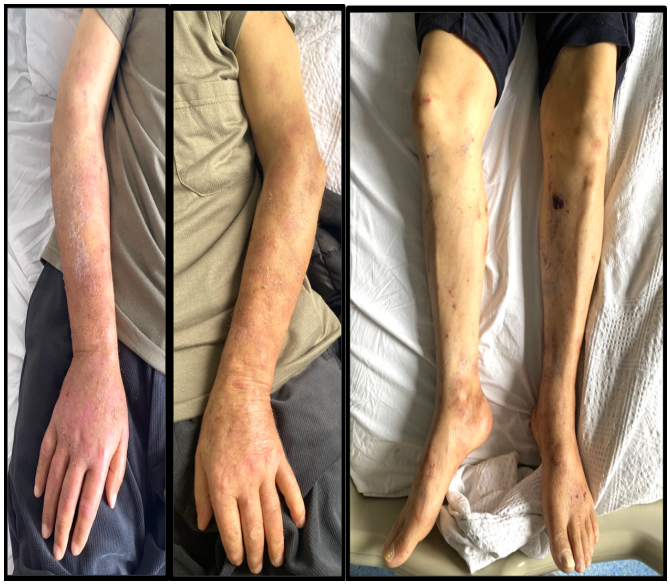

Figure 2. Images of the patient's rash after antibiotherapy (14th day of treatment)

DISCUSSION

The risk of bacterial contamination of blood products is 0.2-0.5%. However, in most of these cases, the number of bacteria is very low, so no clinical findings occur. Although bacterial contamination of blood products is a rare condition, symptoms such as fever, tachycardia, headache, as well as more severe clinical pictures such as septic shock, disseminated intravascular coagulation and death can be seen after transfusion of contaminated products.[2]

There are different results in the literature regarding the frequency of TTBI and the detected agents. The incidence of TTBI is higher in platelet transfusions stored at room temperature than in fresh frozen plasma and refrigerated erythrocyte transfusions.[2] According to German hemovigilance data, in most of the confirmed TTBI cases (72.5%, 29 cases), bacteria with medium or high human pathogenicity such as *Bacillus cereus, Escherichia coli, Klebsiella oxytoca, Klebsiella pneumoniae, Serratia marcescens, Staphylococcus aureus, Streptococcus agalactiae, Streptococcus gallolyticus, Streptococcus dysgalactiae* and *Streptococcus pneumoniae* were detected.[3] In TTBI cases reported to the National Healthcare Safety Network hemovigilance module in the United States between 2010 and 2016, the most frequently detected pathogens were Babesia spp. (16/23, 70%) in erythrocyte concentrates and Staphylococcus aureus (12/30, 40%) in platelet concentrates.[4]

Although bacteria isolated as *s.* were reported in the literature, no case report of *Bacillus thermoamylovorans* bacteraemia was encountered. While there is not enough information in the literature about *Bacillus thermoamylovorans* contaminating blood products, this case may be the first case reported of *Bacillus thermoamylovorans* bacteraemia developed after blood product transfusion.

Bacillus thermoamylovorans was first isolated and described as a gram-positive, moderately thermophilic, facultatively anaerobic, catalase-positive, non-sporulating, rod-shaped and peritrichous flagellated bacterium from palm wine, a tropical alcoholic beverage sampled in Senegal in 1995.[5]

However, a more recent study has shown that this bacterium is actually a spore-forming bacterium.[6]

The bacteria were subsequently isolated from thermal regions, including hot water sources such as spa water.[7] Through their spores that survive at ultra-high temperatures (UHT) and are resistant to pasteurization and heat, these bacteria contaminate milk, causing bad taste and structural defects in milk.[8]

There are case reports about the development of cutaneous infection with some bacillus species.[9,10] There is no case report in the literature showing any association between *Bacillus thermoamylovorans* bacteraemia and development of cutaneous infection/pustular rash.

Blood culture is taken under sterile conditions in our center and the risk of contamination is low. Considering the patient's clinical condition and response to antibiotherapy, contamination was not considered. Since the patient developed deterioration in general condition, fever and rash after transfusion and did not go out of his daily routine except for transfusion recently, the agent grown in the blood culture was attributed to transfusion in the foreground. Attempts were made to contact the transfusion center but without success. The transfused product could not be reached. The significant regression of symptoms and acute phase reactants with appropriate antibiotherapy was considered as strong evidence that the present clinical presentation was related to *Bacillus thermoamylovorans* bacteremia.

Since no transfusion product was found, our case can be considered as a highly probable suspicious TTBI case. The case was registered as a TTBI case by the infectious diseases department and the hematology department.

CONCLUSION

Bacteremia after blood transfusions, although rare, can be seen. This case is presented in order to contribute to the literature in terms of being a case in which *Bacillus thermoamylovorans*, that is a very rare agent in the etiology of both pustular lesions and infections that may develop after blood transfusions, was detected.

ETHICAL DECLARATIONS

Informed Consent

The patient signed and 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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