IJHOSCR

ISSN (P): 1735-1243
ISSN (e): 2008-2207

International Journal of Hematology- Oncology and Stem Cell Research
Vol.4, No. 2, April 2010

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Aims and Scope

International Journal of Hematology-Oncology and Bone Marrow Transplantation has been published since 2004, in hematology and oncology domains especially as the only journal in all stem cell transplantation domains with wide distribution. The journal is publishing in English language.

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The main topics that the journal would welcome are:

Hematology, oncology and stem cell transplantation in all basic and clinical fields

We would be very delighted to receive your original article, review article, commentaries, case report and letter to editor on the above mentioned research fields.

Ardeshir Ghavamzadeh, MD
Professor in Hematology-Oncology
Chairman

IJHOBMT is published quarterly by the Hematology- Oncology and Stem Cell Transplantation Research Center, Tehran University of Medical Sciences in collaboration with Iranian Hematology- Oncology Society and Iranian Bone Marrow Transplantation Society.

Annual subscription rates Rls 240,000 for Iran and US$ 40 for other countries.

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Drug Utilization Review of Conventional Amphotericin B in Febrile Neutropenic Patients Hospitalized at a Bone Marrow Transplant Center

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Abstract

Introduction: Conventional amphotericin B is one of the antifungal choices as prophylactic and empiric treatment against fungal infections in febrile neutropenic patients. However the time of initiation, dosing and monitoring of drug adverse effects must be justified to maximize the efficacy and minimize the toxicities of this antifungal agent.

Methods: We conducted a prospective observational study at Shariati teaching hospital, Hematology – Oncology and Stem Cell Transplantation Research Center, Tehran University of Medical Sciences to evaluate the appropriateness of amphotericin B utilization for our adult bone marrow transplant (BMT) patients for a period of six months.

Results: The charts of a total of 54 patients in 3 adult BMT wards were prospectively evaluated. Most patients underwent allogienic transplantation (61.1%). The mean duration of treatment with amphotericin was 9.3 days with 50% as prophylactic and 42.6% as empiric treatments. Appropriate dose was initiated in 70.4% of patients versus 22.2% unjustified initial doses. The use of amphotericin was appropriate in 92.6% of cases versus 7.4% unjustified uses.

Conclusion: Based on the results of this study, in the majority of our BMT patients amphotericin B was utilized appropriately either as prophylactic or empiric treatment. More attention in dose adjustment seems to be necessary to minimize nephrotoxicity and other adverse effects of this agent.

Key words: Amphotericin B, Febrile Neutropenic, Empiric and Prophylactic Treatment

Introduction

In neutropenic patients, fever can be the only sign of infection however it is not specific symptom.(1) About 60% of febrile neutropenic patients have an established or occult infection and when neurophil counts goes under 100 cells/mm3, about 20% of patients have a bloodstream infection.(2, 3) Bacterial pathogens are considered as early causes of infections in this population whereas fungal pathogens and viruses are common causes of later infections.

The risk of fungal infections in febrile neutropenic patients who have undergone chemotherapy, radiation and bone marrow transplantation (BMT) increases as the length of neuropenia increases. In practice, there are a number of antifungal agents as prophylactic or empiric treatments against fungal infections which include "-azole" family like fluconazole and voriconazole, echinocandins like caspofungin and micafungin, and the class of amphotericins including conventional formulation

of amphotericin B, liposomal formulation and lipid soluble formulation of amphotericin.(4)

Amphotericin B which binds to ergosterol in fungal cell membrane causes the membrane alteration, cytoplasmic leakage and cell death. This fungicidal agent may induce nephrotoxicity and electrolyte imbalance so appropriate initial dose and dose adjustment would be helpful to minimize the adverse effects and toxicities.

In this study we run a drug utilization review (DUR) for amphotericin B in the adult patients who have undergone BMT.

Methods

We conducted a prospective observational study at the Hematology-Oncology and Bone Marrow Transplantation Research Center/Tehran University of Medical Sciences (Shariati hospital). The population from which the participants of the present study were drawn included 54 patients admitted to the adult BMT wards. The charts were reviewed by the staff pharmacists on daily basis for the six-month period. The evaluation forms were developed to ease the data collection based on patients' demographic data, including age, gender, reason and type of transplantation, length of hospital stay, antimicrobial regimen during the hospitalization,vital signs (temperature, blood pressure), kidney function (serum creatinine, creatinine clearance), white blood cell (WBC) counts, microbiology tests including cultures, amphotericin B initial dose, further dose adjustment, duration of treatment, also administration data like amphotericin concentration, pre-medication, pre-hydration, adverse drug reactions related to the infusion or other toxicities including nephrotoxicity and electrolyte imbalance. We also utilized criteria from national comprehensive cancer network (NCCN) for justification of the treatment with amphotericin B (e.g., febrile neutropenia at the time of treatment initiation, positive galactomannan test, febrile after 5 days of antibacterial treatment and prophylaxis with fluconazole).(4)

Results

Fifty four patients in three adult BMT wards were evaluated. The most common reasons for transplantation were acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), and thalasemia, 20.4%, 18.5% and 16.7% of all cases respectively. In our study, the majority of the patients were males (64.8%), in the age group of 20 to 40 years (50%). Table-1 shows the demographic data of 54 patients included in the study. Regarding administration of amphotericin B, the only diluents used was dextrose 5% in water solution with the final concentration of 0.1mg/ml which was compatible with pharmacy references (e.g., American Pharmaceutical Association drug information handbook).(5) Patients hospitalized in all three wards were premedicated with 50 mg intravenous hydrocortisone and a volume between 3-4 litter intravenous fluids as the routine daily hydration .But only in one ward, patients received normal saline contained potassium chloride and magnesium sulfate as pre-hydration before each dose of amphotericin B. Regarding the dosing, 70.4% of initial doses were appropriate whereas 22.2% of the treatment courses initiated with inappropriate dose or inadequate dosing regimen based on renal function indicators. Through the course of treatment, 27.8% of patients had an increase in their serum creatinine while only 3.7% of cases had dose reduction.

According to criteria for evaluation of amphotericin B indications and regarding the nationwide unavailability (highly expensive if any) of intravenous fluconazole to be used as prophylactic treatment in patients with severe mucusitis, nausea and vomiting and also graft versus host disease (GVHD), we found that 92.6% of patients justified utilization, while 7.4% of cases were identified as an inappropriate treatment. Of all cases, 50% of treatments were prophylaxis versus 42.6% empiric treatment courses. Table-2 shows the treatment justification criteria data. The relationship between initial diagnosis and febrile neutropenia is demonstrated as a bar chart in figure 1.

Discussions

Based on the definition of febrile neutropenia in infectious diseases society of America (IDSA) guidelines, fever is defined as a single temperature of $\geq$38.3˚C or sustained temperature of $\geq$38˚C for $\geq$1 hour. A neutrophil count of <500 cells/mm^3 is defined as neutropenia. The lower the neutrophil count and the longer the duration of neutropenia, the higher the risk of infections.(6) Fungal infections are usually secondary after using courses of antibacterial treatment. Studies have shown that about 30% of febrile neutropenic patients who are not responsive to 5-7 days of broad spectrum antibacterial treatments have a systemic fungal infection, mostly caused by Candida or Aspergillus species.(7, 8) Also when the gastrointestinal (GI) mucosa is damaged due to high doses of

chemotherapy or GI- GVHD, patients become predisposed to blood stream Candida infections.(9)
Amphotericin B is an effective option with a broad coverage of both Candida and Aspergillus species (except for Aspergillus tereus which is resistant to amphotericin), although the utilization of its conventional formula is limited due to renal toxicities. Walsh et al, showed the non-inferiority efficacy of liposomal formula and fewer adverse effects in comparison with conventional amphotericin.(10)
In two separate studies conducted by Eriksson et al, and Spiech et al, the teams evaluated the tolerability of 24- hour infusion of conventional amphotericin B and both found the continuous infusion as safe and effective treatment against fungal infections.(11, 12) It is recommended to keep the patient well hydrated during the course of amphotericin therapy to minimize its nephrotoxicity and use pre-medications (steroids, acetaminophen and antihistamines) to minimize its infusion-related adverse reactions.(5) Due to more benign adverse drug reaction profiles of fluconazole and echinocandins (caspofungin, micafungin and anidulafungin), the use of such antifungals are practically seen more common than conventional amphotericin B for either prophylaxis or empiric treatments in neutropenic patients.(13) In fluconazole-treated patients with persistent febrile neutropenia, switching to amphotericin B as empiric therapy seems to be reasonable since the occult fungal infection would be due to fluconazole resistant species.(10) The recommended prophylactic dose of conventional amphotericin B is 0.1 to 0.25mg/kg while the dose of empiric treatment is 0.6 to 1.5 mg/kg. Routine monitoring of renal function and serum electrolytes as well as dose adjustment of amphotericin B if necessary seems to reduce the nephrotoxicites.

Conclusion

As the results of this current study showed, the utilization of amphotericin B either as prophylaxtic or empiric treatment was justified in majority of cases (92.6%) with 50% of cases as prophylactic therapy. Since the average course of therapy is over a week for the majority of patients, it is crucially important to monitor and adjust the renal function and amphotericin dose, respectively. Also, it seems necessary to develop a uniform protocol for both hydration and pre-medication while using this antifungal agent to minimize the infusion-related adverse drug reactions.

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Mir-155 Downregulation by miRCURY LNA™ microRNA Inhibitor Can Increase alpha Chain Hemoglobins Expression in Erythroleukemic K562 Cell Line

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Abstract

Background: MicroRNAs (miRNA) are small non-coding RNAs that have a distinguished role in post-transcriptional gene expression. It's estimated that 10-30% of human mRNAs are regulated by miRNAs. Many miRNAs profiles change during normal erythropoiesis in which some of them are stage specific.mir-155 was downregulated 200 fold in erythropoiesis.

Materials and methods: K562cells were grown in RPMI1640 and viability tested by trypan blue. microRNA 155Inhibitor and its scramble were purchased from exiqon. K562 cells were transfected using transfection kit according to manufacture manual. After RNA extraction and cDNA synthesis, miRNA downregulation confirmed by miRNA Real time PCR, then alpha- and zeta- chain expression was investigated by RT and QRT-PCR.

Results: The viability of cells before transfection was 99% and the efficiency of mir-155 inhibitor transfection was 90%. By relative Q-PCR the zeta chain expression was increased 3.4 fold and alpha chain was increased 8.3 fold in comparison to untransfected cells.

Conclusion: This study showed that mir-155 downregulation has a distinguished role in alpha chain hemoglobin mRNA expression level. The expression of alpha chain was more than zeta chain that may be result of adult source of K562 cells. Differentiation induction by miRNA regulation without adding any growth factor can be considered as a new strategy in gene therapy and tissue engineering.

Key words: Erythroid, Hemoglobin, miRNA, mir-155

Introduction

microRNA (miRNA) are newly discovered non-coding RNA that have a distinguished role in post transcriptional gene expression. These small molecules act by binding to 3 UTR region of target mRNAs and act as fine-tune for regulation of multiple pathway.(1-4)

Each miRNA has many target genes and each gene may regulated by many miRNAs. This network of miRNA action and crosstalk of miRs with transcription factors show a large complicated network of cellular regulation in molecular level.(2)

It's estimated that 10-30% of human mRNAs are regulated by miRNAs.(3)

miRNAs may have tissue specific expression, for example miRNAs 142, 181, 223 expressed mainly in hematopoetic system. Some miRNAs such as mir-17, 24, 146, 155, 128 and 181 have role in early hematopoiesis. Mir-223 is a main miRNA to

promote granulopoiesis by blocking NFI-A and increasing of C/EBP-α transcription factors(4 -6)

About 19 miRNAs down regulated during Megakaryopoesis including miR-10a, 10b, 30c, 106, 126, 130a, 32, 143, miRNAs 223, 15a, and 16-1, exhibited a biphasic expression pattern; suggesting a stage-specific function.(7)

Many miRNAs profiles change during normal erythropoiesis that some of them are stage specific. For example mir 155, 221, 223 downregulated (200, 20, 10 fold respectively) and mir-451 upregulated 270 fold in erythrocyte differentiation.(2, 6, 8) Perhaps down-regulation of miR-221 and miR-222, unblocks KIT expression and allows erythroblast expansion.(7)

Yang used miRNA array to characterize miRNA variation of K562 cells before and after hemin treatment and showed that miR-126 exhibited up-regulation while miR-103, miR-130a, miR-210, and miR-18b exhibited down-regulation after hemin induction.(9)

Zhan et al analyzed more than 295 miRNA pofiles in erythroid differentiation by microarray. They showed that more than 100 miRNA expressed in this lineage. Also, they show similar up-regulation of miR-451, miR-24, and miR-16 and down-regulation of miR-221-222 and miR-155 during erythropoiesis.(10)

Human mir-155 is predicted based on homology to a cloned miR from mouse, Lagos-Quintana eta la described the identification of 34 novel miRNA by tissue- specific cloning in mouse. They found mir-155 (UUAAUGCUAAUUGUGAUAGGGG) in colon tissue.(11) In 2004, Kasashima et al investigated miRNAs during 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced differentiation of human leukemia cells (HL-60) into monocyte/macrophage-like cells. They found three novel and 38 known miRNAs expressed in HL-60 cells, and experimentally validated mir-155 in human HL-60 leukemia cells.(12)

Like the mouse miRNA, human mir-155 resides in the non-coding BIC transcript, located on chromosome 21. In most cases, sequences of the new mature miRNAs were perfectly conserved between human and mouse, in mir-155 case the mature form differs from that in the mouse at a single position.(13)

Eis et al. confirm that miR-155 is processed from the BIC transcript in human, and demonstrate elevated expression of miR-155 in lymphoma samples including diffuse large B cell lymphoma (DLBCL).(14)

Landgraf in collaboration with more than 20 research centers comprised miRNA expression in the hematopoietic system to all other organ systems and showed that only 5 miRNAs are highly specific for hematopoietic cells: miR-142, miR-144, miR-150, miR-155, and miR-223.

The mature sequence of mir -155 (MI0000681= CUGUUAAUGCUAAUCGUGAUAGGGGUUUU UGCCUCCAACUGACUCCUACAUAUUAGCA UUAACAG) that shown in databases represents the most commonly cloned form from Mammalian microRNA Expression Atlas Based on Small RNA Library Sequencing.(15)

While mir-155 is highly expressed in human cord blood derived hematopoetic stem cell, many reports indicate that miR-155 is down-regulated during erythropoiesis induction in primary cells or cell lines.(2, 16)

In 2009, Faraoni et al, presented mir-155 as a multifunctional microRNA. They reviewed this miRNA role in hematopoiesis, inflammation, immunity, neoplastic disease, leukemia, solid tumors, cardiovascular disease and viral infection.(17)

K562 cell line is bcr-abl positive and possesses erythroid features. Different clones of these cells express Hb Portland (ζ2γ2), Gower1 (ζ2ε2), Gower2 (α2ε2) and small quantities of Hb F (α2γ2). Although initial clones of K562 cells have shown heterogeneity in hemoglobin content. Furthermore, as a result of previous reports erythropoiesis inducer cannot induce synthesis of ß chain.(18)

So, this cell line was selected as a model for evaluation of mir-155 downregulation effect on α- and ζ- chain hemoglobin mRNA expression.

Materials and methods Cell culture: This cell line is supplied by the National Cell Bank of Iran, Pasteur Institute, Tehran, Iran. K562cells were grown in RPMI1640 medium (Gibco, USA) with 10% fetal Bovin serum (FBS) (Gibco,USA), 1X Penicillin sterptomycin antibiotic and 2mM L-glutamin at 37ºC under a humidified atmosphere consisting of 95% air and 5% CO2 for 7 days. On the day of transfection, cells were counted and tested for viability by trypan blue. Then in 6 -well format plated, 2×10^5 cells per well in 3 ml, complete growth medium.

miRCURY LNA™ microRNA Inhibitor and Scramble: miRCURY LNA™ microRNA Inhibitor purchased from exiqon(EXIQON, Denmark). This product is based on the LNA™-technology for superior specificity and biostability.

Locked nucleic acid (LNA™) nucleosides are a class of nucleic acid analogues in which the ribose ring is "locked" by a methylene bridge connecting the 2'-O atom and the 4'-C atom. LNA™ nucleosides contain the common nucleobases (T, C, G, A, U and mC) and are able to form base pairs according to standard Watson-Crick base pairing rules. However, by "locking" the molecule with the methylene bridge the LNA™ is constrained in the ideal conformation for Watson-Crick binding. K562 cells were transfected using Lipofectamine 2000 (Invitrogen, USA) according to manufacture manual, briefly one day before transfection 2×10^4 cells were plated in 2.5 cc medium with 10% FBS without antibiotic. In one tube 100 pmol mir-155 inhibitor was diluted into 250µl OPTIMEM (Gibco, USA). In another tube 5µl lipofectamin 2000 were diluted in 250µl optimum. After a 5 -minute incubation, two tubes combined and incubated for 20 minutes. Finally, this compound added to cultured plates. Transfection of scramble was done, similarly. Scramble was used for the evaluation of transfection efficiency and non-specific effects of oligonucleotides. Every 4 days, these stages were repeated. All tests were done in triplicate manner.

RNA extraction and cDNA synthesis: Total RNA was extracted from 2×10^5 cells (from 6 -well culture plate) of controlled, scrambled and anti-sense treated samples by using Biozol reagent in third and seventh days after starting of transfection. Briefly, 500µl biozol was added to counted cell and followed by phenol/cholorophorm precipitation according to the manufacture instructions. The purity examination of extracted RNA was done by spectrophotometrically at 260/280nm and gel electrophoresis.

In order to gain maximum purity, extracted RNA was treated by DNAse I (fermentas) as manufacture guidelines.

cDNA was synthesized by cDNA synthesis kit (Bioer). In sum, 20 ng of total RNA was reverse transcribed in a total volume of 20µl containing oligodt 18 primer 1 µl, AMV enzyme 0.5 µl, 5x buffer 4 µl , RNase Inhibitor 0.5 µl, dNTP mix 1 µl, RNase -free watern: up to 20 µl as recommended by manufacturer.

miRNA Real time PCR: In this study third day extracted RNA, was used for miRNA assay. The high-specificity miRNA QPCR Core Reagent Kit provides the reagents for quantitative PCR amplification of cDNA templates derived from miRNAs within a total RNA population. Because of their short length, miRNAs are difficult to detect with standard QRT-PCR protocols. As a first step, use the miRNA 1st-strand cDNA synthesis kit (Stratagene, USA) to elongate miRNAs in a polyadenylation reaction and then reverse transcribed the polyadenylated RNA into QPCR-ready cDNA. The target of interest is then amplified and detected using the high-specificity miRNA QPCR core reagent kit (Stratagene, USA). The universal reverse primer serves as the downstream primer in the QPCR reaction, the specificity of the QPCR reaction is provided by the miRNA-specific forward primer. Our forward primer for mir-155 was TTAATGCTAATCGTGATAGGGGT.

Real time PCR: Quantitative RT-PCR was carried out using SYBER GREEN master mix (Bioer), Primers α chain Forward CCGACAAGACCAACGTCAAGG α chain reverse GGTATTTGGAGGTCAGCACG,ζ chain Forward GGTGAAGAGCATCGACGACA ζ chain reverse TCTCGGTCAGGACAGAGGA, GAPDH forward GACAAGCTTCCCGTTCTCAG, GAPDH reverse GAGTCAACGGATTTGGTCGT with a thermal cycler BIOER according to manufacture protocol (94˚C 2min, 94˚C 10sec, 58˚C 15sec, 72˚C 25sec) in 40 cycles.

The alpha- and zeta- chain expression were normalized with GAPDH internal control and the relative gene expression was calculated using Ct ($2^{-\Delta\Delta ct}$) method.

Results

Cell expansion and cell transfection: K562 cell line was suspained and passaged in RPMI 1640 with 10% fetal Bovin serum (FBS), 1X Penicillin sterptomycin antibiotic and 2mM L-glutamin. On transfection day, cells were counted and tested for viability. The viability of cells before transfection was 99%.

Cells in log phase were selected for mir-155 inhibitor transfectin. Transfection was done in duplicate but before the assay, cells were mixed. After overnight incubation, medium was changed and cells were evaluated for determination of transfection efficiency. Since miRCURY LNA™ microRNA Inhibitor and its scramble were FITC-labeled, transfection efficiency was measured by flow cytometery. The mean transfection efficiency in our experiments was about 60%.

miRNA 155 downregulation confirmation: To exclude mir-155 changes after transfection short RNA U6 was selected as a normalizer for

measuring the content of mir-155. miRNA real time PCR was done on miRNA- specific cDNA synthesized from third day sample after starting transfection. Analysis of result, showed about 20 fold reductions in the expression of mir-155. The trasfection was repeated each three day for the permanent suppression of mir-155.

Reverse transcriptase PCR on alpha chain hemoglobins

RT-PCR analysis showed that transfected K562 cells with mir-155 inhibitor express both α and ζ hemoglobin chains.

Figures- 1, 2 show alpha- and zeta- chain expression on 3% agarose gel dyed with ethidum bromide indicating these transfected cells express both primitive and definitive hemoglobins.

Alpha and Zeta chain quantitative real time-PCR: By relative Q-PCR, the zeta chain expression was increased 3.4 fold and alpha chain was increased 8.3 fold in comparison to untransfected cells. The effect of scramble on alpha and zeta chains expression was not statistically important. (P>0.05), (Figure- 3)

Conclusion:

Red cells are the most abundant cell type in the human body, with more than 25 trillion in the bloodstream.

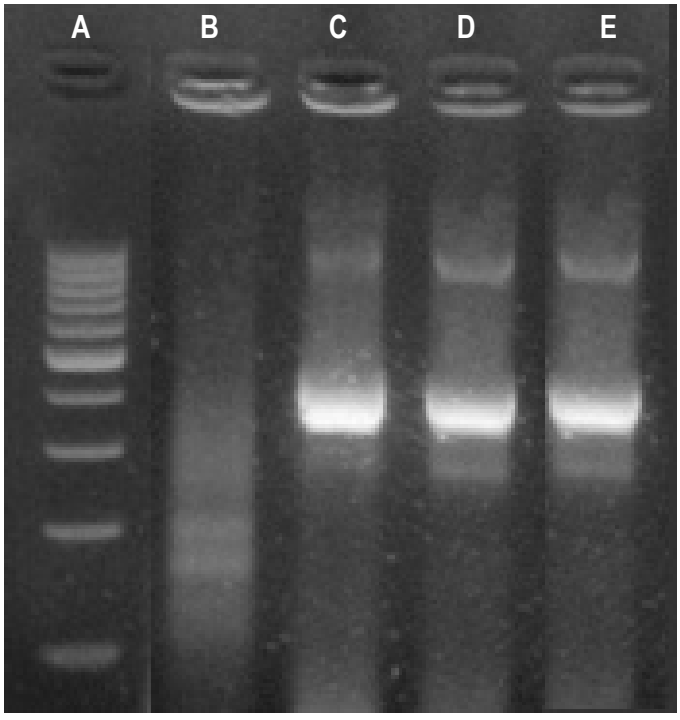

Figure 1: Alpha chain RT-PCR A: Ladder 100 bp; B: NTC (non template control); C: mir-155 inhibitor- treated cells; D: scramble- treated cells; E: untransfected control cells (product size: 407bp)

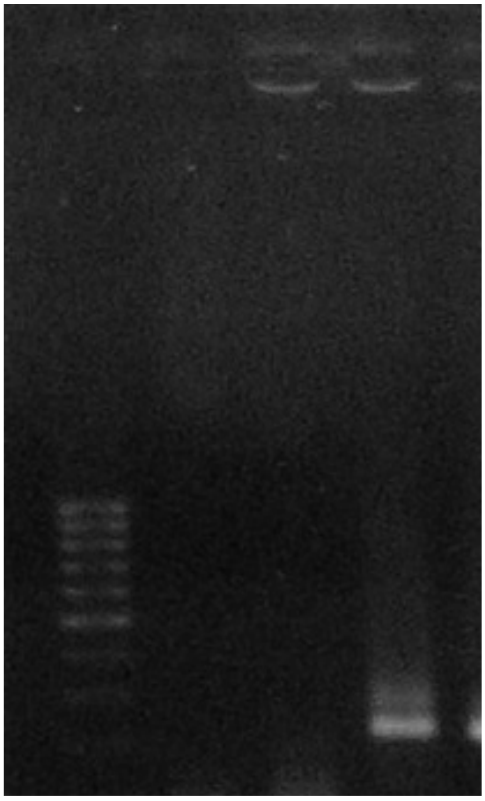

Figure 2: Zeta chain RT-PCR A: Ladder 100 bp; B: NTC (non template control); C: NPC (non-primer control); D: untransfected control cells; E: scramble- treated cells; F: mir-155 inhibitor- treated cells (product size: 208 bp)

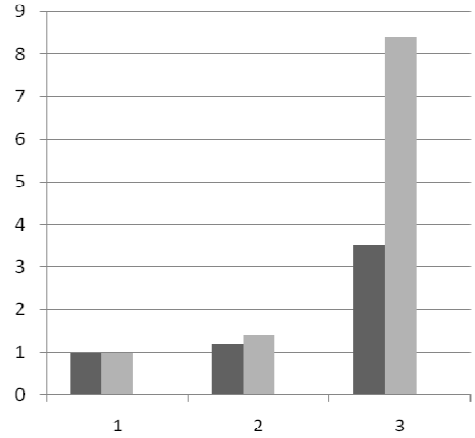

Figure 3: Effect of miR-155 dow-nregulation on erythroid differentiation of K562 cells. The anti-miR155 and scramble were transfected into untreated cells .The cells were assayed for gene expression of α and ζ chains at 7 days post- transfection. Expression of these chains was detected by qRT-PCR. Relative fold changes of gene expression were calculated by ΔΔCt method and the values are expressed as $2^{-\Delta\Delta CT.}$ Data represent the mean plus standard deviation of three independent experiments. The statistical significance between NC/Cy3 control and particular samples was calculated by Student's t-test ($p < 0.05$). 1: untreated cells; 2: scramble treated cells; 3: mir-155 inhibitor treated cells. Dark bar shows ζ gene expression and gray bar indicates α gene.

Red cells are produced in the bone marrow where they undergo progressive maturation from unilineage progenitors to morphologically defined precursors to enucleated erythrocytes.

Erythropoietin is the primary cytokine regulating erythroid cell maturation but other factors such as testosterone, estrogen, GM-CSF, IL-9, IL-3 and SCF are involved. Erythropoiesis is also regulated by transcriptional complexes containing GATA-1, SCL, EKLF, and multiple other factors.(19)

MicroRNAs have recently been found in erythroid cells and raise the possibility that gene downregulation is also important for lineage maturation.

microRNA (miRNA) are non-coding RNA that have a distinguished role in post- transcriptional gene expression. These small molecules act by binding to 3 UTR region of target mRNAs and act as fine-tune for regulation of multiple pathways such as proliferation, differentiation, apoptosis and cancer.

Recently multiple miRNAs are known to be involved in erythropoesis. miRNAs such as mir-451, mir144 and mir-210 are up -regulated in erythroid differentiation process but mir-155, mir-150, mir-221, mir-222, mir-223, mir-24 are down-regulated.

Mir-155 is a multifunctional miRNA that plays crucial role in various physiological and pathological pathways such as hematopoesis, inflammation, immunity and cancer.(17)

In this study we showed that mir-155 downregulation by antisense can increase α and ζ chains expression in K562 cells without any erythropoesis induction agent and cytokines such as hemin, DMSO, EPO and TPO.

Each miRNA has several targets and each gene has several biding sites for multiple miRNAs, so it's not far-fetched that manipulation in one or more miRNA profiles can start a cascade of events resulting in differentiation, proliferation, and apoptosis in cells.

Several studies have shown mir-155 down-regulation in the process of erythroid differentiation.(2, 3, 8, 10) Our present study is fully compliant with previous reports.

In 2007, Masakai et al, showed that the expression level of miR-155 decreased about 200-fold, and the expression of miR-451 increased about 270-fold within 12 days of cultures during normal human erythropoiesis. They introduced mir-451 and mir-155 as a key molecule in erythroid differentiation. Our manipulation in mir-155 level which resulted in an increased alpha- and zeta- chain expression is a confirmatory note on their result.(2)

Bianchi and colleagues carried out complete analysis on 194 expressed miRNA during erythropoesis and observed that their findings are completely compliant with previously reports. According to their report miR- 15b, miR-16, miR-22, miR-185, miR-181, mir-150, mir-155, mir-221, miR-222, miR-223, mir-451, miR 144, miR-188, miR-362 and miR-210 are changed during erythropoiesis. So, our finding about mir-155 doesn't have any incompatibility with their result.(3)

The alpha globin gene cluster is on chromosomes 16. This cluster has an upstream H40 regulatory region and ζ, α2 and α1 globin chain genes; there are two pseudogenes, ψ and ζ.

According to TESTA et al, report K562 clones are divided into three groups: in group I no hemoglobin can be detected but in group II Hb Portland and in group III Hb Portland and Gower I are present.(18) It should be noted that α chain synthesis was occasionally present in minute amounts of K562 clones and mir-155 suppression by antisense, increased vigorously α -chain expression in comparison with ζ chain.

Since, miRNAs are negative regulators in post-transcriptional level of gene regulation, likely our induced down-regulation of mir-155 unblock hemoglobin gene expression.

In 2007, Cavazzana-Calvo and colleagues treated an 18-year-old male patient who had HbE/β-thalassaemia. They treated the patient's HSCs with an HIV-derived lentiviral vector containing a functional β-globin gene after high dose of chemotherapy.

The HSCs containing the transferred β-globin gene gradually gave rise to healthy blood cells and levels of the normal β-globin protein increased; this resulted in the improved production and quality of red blood cells.(20)

Designing a same study with replacing globin genes responsible for miRNAs instead globin genes maybe facilitate and make faster thalassmias and hemoglobinopathies gene therapy pathways.

A better understanding of the regulation of the globin genes expressed in the embryo, fetus, and adult will ultimately lead to improved therapies for people with hemoglobinopathies, including sickle cell disease and thalassemia. Differentiation induction by miRNA regulation without adding any growth factor can be considered as a new strategy in gene therapy, regenerative medicine and tissue engineering.

Acknowledgements

We appreciate the assistance of Dr. Abroun in performing this research. We acknowledge the

financial support provided by Tarbiat Modares University, Tehran, Iran.

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Comparisions of ARMS-PCR and AS-PCR for the Evaluation of JAK2V617F Mutation in Patients with non-CML Myeloproliferative Neoplasms

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Abstract

Background and objectives: JAK2 is a nonreceptor tyrosine kinase that plays a major role in myeloid disorders. JAK2V617F mutation is characterized by a G to T transverse at nucleotide 1849 in exon 12 of the JAK2 gene, located on the chromosome 9p, leading to a substitution of valine to phenylalanine at amino acid position 617 in the JAK2 protein. In this study we compared two molecular methods namely ARMS-PCR and AS-PCR for the evaluation of JAK2V617F mutation in patients with myeloproliferative neoplasms.

Material and methods: In this study we evaluated JAK2 mutation in 89 patients with Myeloproliferativeneoplasm (MPNs) by simple randomized sampling. The mutation was detected by ARMS-PCR and AS-PCR in patients.Three DNA samples were sequenced for conformation of the above techniques.

Results: The JAK2 V617F mutation was detected in 86.6% (26/30) of patients with polycythemia vera and 61.5% (8/13) of patients with idiopathic myelofibrosis. None of 31 CML patients were detected by ARMS-PCR and AS-PCR. In essential thrombocythemia using ARMS-PCR and AS-PCR 46.6% (7/15) and 53% (8/15) of patients were positive, respectively. The mutation was confirmed by sequencing.

Conclusions: The results of the study showed that similarity with other studies by two techniques and detection of the JAK2V617F mutation may depend on the molecular technique used. Also, JAK2 mutation detection is an appropriate tool for differential diagnosis of non-CML myeloproliferative neoplasms from benign condition like reactive erytrocytosis and thrombocytosis.

Key words: JAK2V617F mutation, myeloproliferative neoplasms, ARMS-PCR, Allele-specific PCR

Introduction

JAK2 V617F mutation in chronic myelo proliferative neoplasm was discovered by different methods.(1, 4) JAK2 is a cytoplasmic tyrosin kinase with a key role in a different signal transduction pathways like interlukin 3, 5 (IL_3, IL_5) and granolocyte-monocyte-colony stimulation factor (GM-esf).(5, 6) JAK2 contains four major function domains, one tyrosin kinase domain (JH1), one psudo kinase domain(JH2), one sic-homology2 (SH2) domain and the FERM domain.

Identification of the JAK2 mutation is new discovery in the field of chronic myeloproliferative neoplasm-(MPNS).(7, 8) Different techniques have been used for the detection of this mutation, such as genomic DNA-PCR-sequencing, RT-PCR, PCR-ARMS (amplification refractory mutation system), Allele-specific PCR, PCR restriction analysis, and real-time PCR.(9) In this study frequency of JAK2 mutation, in patients with essential thrombocythemia (ET), polycythemia vera (PV) and idiopathic myelofibrosis (IMF) and chronic myelogenos leukemia (CML) was studid using two methods (i.e. AS-PCR and ARMS-PCR) in order to find the most sensitive method for the detection of this mutation.

Materials and Methods

We evaluated 89 previously treated and newly diagnosed patients with MPDs and 50 normal control samples: 30 patients with PV, 13 patients with IMF, 15 patients with ET and 31 patients with CML. Fifty patients were male (56.6%) and 39 were female (43.4%). The mean age was 48 ranging from 16 to 76 years.

The patients were selected from outpatient clinic of Bone Marrow Transplantation Center of Shariati Hospital and the Oncology Center of Imam Khomeini Hospital. The mutation was detected by two methods, AS-PCR and ARMS-PCR on mononuclear cell.(9)

1-AS-PCR method: SsRNA was extracted from 5ml of fresh peripheral blood in EDTA with standard trizol method.(11)

Extracted ssRNA was measured by OD, absorbance in 260nm and ssRNA concentration was calculated using the formula below:

[SSRNA](mg/ml)= A260×40 mg/ml×dilution factor

AS-PCR is a common PCR technique widely used to detect known mutation based on the amplification of mutant alleles using mutant specific primer. This method was first reported by baxter et al.(10) and sequences of the primers used in this study are given below:(10)

Forward 5'-GAAGATTTGATATTTAATGAAAGCCTT-3'

Reverse 5'-GTAATACTAATGCCAGGATCACTAAGTT-3'

Mutant 5'-AGCATTTGGTTTTAAATTATGGAGTATATT-3'

PCR parameters were as follows: Denaturation for 5 minutes at 95ºC, for one cycle; 36 cycles of denaturation at 95ºC for 30 seconds, annealing at 56ºC for 40 seconds, elongation at 72ºC for 45 seconds, hold for 10 minutes at 72ºC, and infinite hold at 4ºC.

A 488-bp product was obtained from both wild type and mutant alleles, while a 295 bp product indicated the presence of the mutation allel.(4) Of course the AS-PCR was not designed to distinguish zygosity. The zygosity was determined by RFLP-PAGE (restriction fragment length polymorphism/ plyacrilamide gel electrophoresis) sample interpretation.(11)

2-ARMS method: Genomic DNA was extracted from 5ml of fresh peripheral blood in EDTA using proteinase K method. Primers used in ARMS-PCR-method include:

Forward Outer (FO): 5'–TCCTCAGAACGTTGATGGCAG-3'

Reverse Outer (RO): 5'-ATTGCTTTCCTTTTTCACAAGAT-3'

Forward wild-type specific (FWt): 5'-GCATTTGGTTTTAAATTATGGAGTATATG-3'

Reverse mutant-specific (RMt): 5'-GTTTTACTTACTCTCGTCTCCACAAAA-3'

We used 25ng of genomic DNA, 0.5µl of each FO, RO and FWt primers and 1µl of RMt primer. DNA was amplified in a 40-cycle PCR reaction. The PCR products were analysed on 3% TBE agarose gels. The FO and RO primers would amplify a 463-bp product from JAK2 gene, while the primer RO and FWt primers would amplify a 229-bp product as wide type alleles and also amplify a 279-bp product from FO and RMt primers.

Results

In this study we compared the results of two methods, ARMS-PCR and AS-PCR.

Result of ARMS-PCR showed that JAK2 mutation was positive in 26 out of 30 PV patients (86%), 8 out of 13 IMF patients (61%), 7 out of 15 ET patients (46.6%) and none of the 31 CML patients (Figure- 1, Table-1). Using AS-PCR method, we observed that 26/30 PV patients (86%), 8/13 IMF patients (61%), 8/15 ET patients (53%) and also none of 31 CML patients were positive for JAK2 V617F mutation (Figure- 2, Table-1).

All mutations were confirmed by sequencing purified PCR product by Millegen Company (Germany).

Table- 1. Result of ARMS-PCR and AS-PCR methods

Disease	AS-PCR	ARMS-PCR
	JAK2V617F positive	JAK2V617F positive
PV	86%	86%
ET	53.3%	46%
IMF	61.5%	61.5%
CML	14%	14%

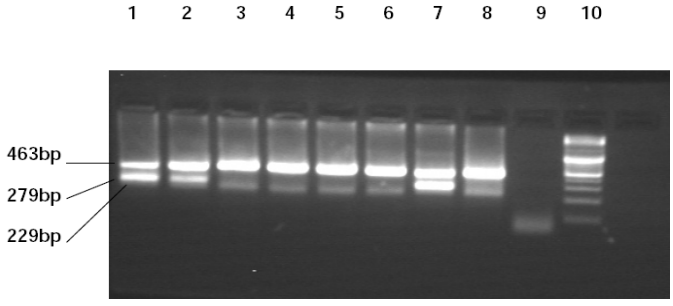

Figure- 1. ARMS-PCR assay for detection of JAK2V617F mutation; lane1, 2 positive control, lane3, 4, 5, 6, 8 mutation negative patients, lane7 mutation positive patients, lane 9 negative control, lane 10, 50bp DNA ladder.

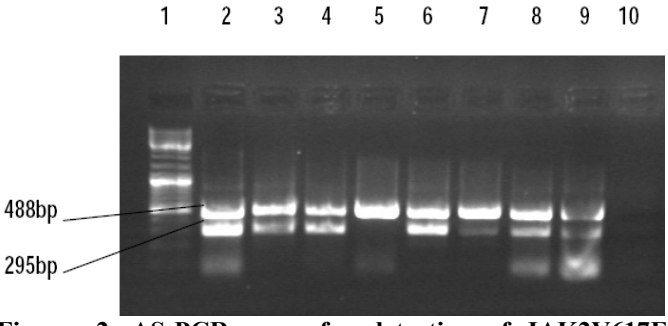

Figure- 2. AS-PCR assay for detection of JAK2V617F mutation; lane1, 100bp DNA ladder, lane2, positive control, lane3,4,6,8 mutation positive patients, lane5 mutation negative patients, Lane10 negative control.

Discussion

JAK2 V617F mutation, in chronic myeloproliferative neoplasm, was investigated by different methods.(1&4) Identification of the JAK2 mutation is new discovery in the field of chronic myeloid proliferative neoplasm (MPNS).(7&8) Different techniques have been used for this detection of the mutation, such as genomic DNA-PCR-sequencing, RT-PCR, and ARMS-PCR, AS-PCR, PCR restriction analysis, and real-time PCR.(9)

In one study, the prevalence of mutation in PV patients has been found to be 81% (58/72), in ET patients 41% (24/59) and in IMF patients 43% (15/53).(11) In Baxter et al'st study in 2005 using mutation specific PCR method,(4) the JAK2 mutation detected in 71 of 73 (97%) patients with PV, 29 out of 51 (57%) with ET, and 8 out of 16 (50%) with IMF.

In support of previous reports, we observed the mutation in 86% of PV patients which is comparable with result of James' (i.e. 86%),(1) Jelinek's (86%) (14) and Jones's studies (81%).(11)

The highest rate of mutation (97%) has been reported by Lippert's group using allele-specific quantitative polymerase chain reaction (qPCR) (15) and the lowest one (65%), has been reported by Kralovics's group using DNA sequencing and microsatelite mapping.(2) The highest detection rate in IMF was found by Jelink (14) with a frequency 95% (18 of 19 patients) who used pyrosequencing method and the lowest observation of mutation with 35% frequency (16 of 46 patients) detected in Levin study.(14) In a study by Campbell et al (25) on 806 ET patients by AS-PCR techniques, 414 cases (53.4%) were positive and 362 (46.6) were negative for this mutation.

Comparisons of the sensitivity of the different techniques for JAK2V617F mutation, show that AS-PCR techniques compared to other methods like RFLP, pyrosequencing and ARMS are more sensitive.(13) The JAK2 mutation has not been found in our 31 CML patients, which is the same as Jones' group study,(13) however, in other study JAK2 mutation and abl-bcr translocation were detected in some patients who had received Imatinib treatment.(16) Moreover, the JAK2 mutation was detected by ARMS-PCR. This result shows that AS-PCR is more sensitive than ARMS-PCR. Probably, the main cause of this difference that we observed between the two molecular techniques is the sensitivity of this method.

In summary, we have shown that a single acquired point mutation in JAK2 is present in virtually most patients with PV and in about half of those with either ET or IMF. Thus, the detection of mutation could be used not only as a diagnostic tool, but also for the classification and management of patients with MPDs. Also, there were no significant differences between the two methods and objectively the AS-PCR is a little more sensitive than ARMS-PCR.

Acknowledgment

We gratefully express our appreciation to all colleagues in Hematology-Oncology and BMT Research Center at Shariati Hospital, Tehran University of Medical Sciences for their scientific and technical assistances.

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PCR-RFLP Is a Useful Tool to Distinguish between C. Dubliniensis and C. Albicans in Cancer Patients in Iran

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Abstract

Introduction: Candida dubliniensis and C. albicans are very similar in morphology and phenotypic characteristics. Approximation of this yeast species has caused major problems in identifying these two correctly.

Materials and Methods: To distinguish among sixty yeast clinical isolates from patients with cancer, polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) was done.

Results: PCR-RFLP of the ITS region showed different patterns between Candida dubliniensis and C. albicans after digestion with enzymes BlnI. All of the Clinical isolates were diagnosed as C .albicans. The results were confirmed by sequencing and RAPD-PCR.

Conclusion: PCR RFLP would be a useful and applicable technique in clinical laboratories for discrimination of C. albicans and C. dubliniensis.

Keywords: PCR- RFLP, C .dubliniensis, C. albicans, BlnI

Introduction

One of the important issues in routine laboratories of medical mycology is the correct identification of C. albicans and C. dubliniensis together. C. dubliniensis is not detectable than yeast species of C .albicans morphology and phenotypically.

They are different in their epidemiology, virulence and antifungal susceptibility. Approximation of this yeast species has caused major problems in identifying the C. albicans correctly. Like C. albicans, C. dubliniensis can produce germ tubes and chlamydospores. These two species can be distinguished by examination of their phenotypic features, including growth at 45°C, intracellular β-D-glucosidase activity, and carbohydrate assimilation profiling.(1,2) However, these phenotypic tests are usually time-consuming and do not give completely reliable results.(2) In spite of the identified differences, a rapid and accurate discrimination between C. albicans and C. dubliniensis remains problematic in the most of clinical mycology laboratories.(3) Due to bugs in the phenotypic methods to identify C. albicans and C. dubliniensis recently, trends have increased about the use of molecular techniques. These techniques, are including 25S rDNA analysis(4) PCR-fingerprinting(3) fluorescent probe hybridization(5) amplified fragment length polymorphism.(6) However, these techniques often are laborious, time consuming and too expensive for routine use in medical laboratories. Mirhendi et.al,(2) introduced a simple polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) for differentiation between C. albicans and C. dubliniensis. The aim of this study is to distinguish C. albicans and C. dubliniensis in clinical yeast isolates from patients suffering cancer using PCR-RFLP.

Materials and methods

Sixty clinical isolates which originated from, lip, throat and tongue of patients suffering from cancer in four Mazandaran University Hospitals -Iran and two standard strains (C .albicans CBS 562; C. dubliniensis CBS 7987) which obtained from CBS-KNAW, fungal biodiversity centre, Utrecht, Netherlands were included in present study. Table one summarized the distribution of these isolates based on the origin of the specimens and type of malignancies. Primarily, these strains were identified by some phenotypic methods such as colony color on CHROMagar Candida medium (CHROMagar Company, Paris, France), germ-tube formation in horse serum, chlamydospore formation on cornmeal agar (DIFCO laboratories, Detroit, Mich., USA) with 1% Tween 80 (CMA-T_{80}) and some other molecular methods such as RAPD-PCR(7, 8) and sequencing for D1/D2 region of LSU rDNA gene.(9)

Stock cultures were initially grown on Sabouraoud's dextrose agar (LAB M, Bury, UK) at 32°C for 48 h, and then differentiated using RFLP PCR technique.

DNA Extraction: genomic DNA extracted using the method of glass bead disruption.(10) Briefly, a loop full of fresh yeasts was suspended in 300µl of lysis buffer (10mM Tris, 1mM EDTA pH 8, ify ITS domains.

PCR amplification was performed in a final volume 50 µl .Each reaction consists of 2 µl template DNA, 0.5 µl of each primers at 25 µM, 1.25 µl of dNTP (BIORON GmbH, Germany) at 5mM, 0.5U Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim,Germany) and 5µl 10×PCR. The amplification condition consists of 35cycles of denaturation at 94°C for 1min, primer annealing at 56°C for 1min, extension at 72°C for 1min. In the first cycle, the denaturation step was 94°C for 5min and in the final cycle the final extention step was 72°C for 7min. Amplified products were visualized by 1% agarose gel electrophoresis in TBE buffer. Gel was stained with ethidium bromide (0.5 µg/ml) and photographed by ultra violet photography.

Restriction Enzyme analyses: ITSI region sequences of several C. albicans and C. dubliniensis were received from NCBI's GenBank. The restriction sites of various restriction enzymes were determined by CLC workbench software (version 3) and the enzymes BlnI; (Roche diagnostics, Swiss) were selected which was similar to Mirhendi et al. study.(2) Digestion was performed by incubation 8.5 µl of PCR products with 0.5 µl of enzyme at 1%SDS, 100mM NaCl, 2% Triton X-100), then 300µl of phenol-chloroform(1:1) solution and 300mg of 0.5 diameter glass bead were added to samples. For disrupting cell, samples were vortexed vigorously for 5min. Then, samples were centrifuged at 10,000 rpm for 5min, supernatant was separated and transferred to a new micro tube and equal volume of chloroform was added, centrifuged at 10,000 rpm for 5min and supernatant was separated and transferred to a new micro tube again. For alcohol precipitation, 2.5 volume of cold absolute ethanol were added and frozen in -20º C for 10 min. After freezing, samples were centrifuged at 12,000 for 12min. The precipitate was centrifuged and washed with 70% ethanol, air-dried, re-suspended in 100µl of TE (10mM Tris, 1mM EDTA) and was preserved at -20ºC until use. DNA was visualized by electrophoresis on 1% agarose gel stained with ethidium

PCR Conditions: PCR amplification of ITS1-5.8S -ITS2 rDNA spacers, was achieved using the ITS1 (forward, 5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (reverse, 5′-TCC TCC GCT TAT TGA TAT GC -3′) primer pairs (MWG-Biotech AG, Germany) to ampl

10U in a final reaction volume 0f 10 µl at 37ºC for 3 h. Restriction fragments were separated by 3% agarose gel in TBE buffer for 1h at 100 V. Gel was stained with ethidium bromide (0.5 µg/ml) and photographed by ultra violet photography.

Results

In this study, we apply a PCR-RFLP for distinguishing between C. dubliniensis and C. albicans using the universal primers ITSI and ITS4 for amplification the ITS region. The universal primer pair, ITS1 and ITS4 amplified DNA from all 60 clinical isolates and two standard strains and showed a unique band approximately 535bp. When the PCR products from ITS1/ITS4 amplifications were digested with BlnI enzyme C. dubliniensis and C. albicans showed different patterns of DNA fragments. The C. dubliniensis digestion produced two strong bands of about 200bp and 335bp and there were no cutting sites in C. albicans thus, only one fragment that has the same size as PCR has been created (535bp).(Figure- 1 and Table- 2)

PCR-RFLP analysis showed that all of the clinical strains were C. albicans. Results on the ITS region DNA sequence analysis did not show sequence variations comparing with NCBI GenBank and the

results were compatible with profiles which generated for the same species using RAPD(7) and had similarity when compared with two standard strains also.

Discussion

Opportunistic fungi are life-threatening infections in immunocompromised patients.(11) In recent years, Candida species have emerged as the major hospital pathogens.(12**)** Although Candida albicans remains the most frequently cause of Candidiasis, the incidence of the disease caused by other species of Candida that are less sensitive to azoles compounds has increased steadily.(11) Nowadays**,** Candida species have been identified based on routine laboratory techniques such as germ tube formation and bio chemical tests. These procedures require purification of the target organisms which are laborious and time-consuming and may not be species specific also.(13) Due to the high degree of phenotypic similarity between C. albicans and C. dubliniensis, accurate and rapid identification in routine laboratories remain problematic.(3) In this study, we apply a PCR-RFLP method using the universal primers ITS1 and ITS4 to amplify the ITS1 and ITS2 region and 5.8S in the rDNA gene and identify Candida species of suspected of C. albicans. Primarily, these strain were identified by phenotypic methods color of colony on CHROMagar Candida medium (CHROMagar, Company, Paris, France), germ-tube formation in horse serum, chlamydospore formation. Strains were identified by RAPD-PCR and sequencing definitively. Some of these strains were determined for D1/D2 region of LSU rDNA gene.(7-9) Several studies using PCR techniques together with restriction digestion enzymes for special identification of species have also expressed several techniques with universal primers for identification of various fungi have been reported.(2,14-17)

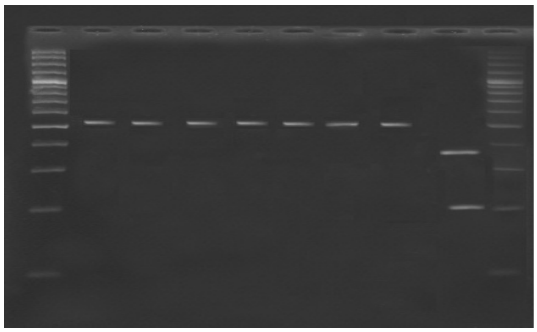

Figure- 1. Lane 1: molecular size marker (100bp). 2, 4, 6: PCR product of C.albicans. 3,5,7: digestion of PCR product of C. albicans with BlnI. 8: PCR product of C. dubliniensis. 9: digestion of PCR product of C. dubliniensis with BlnI. Lane 10: molecular size marker (100bp).

These primers have already demonstrated their efficiency.(2,14-17) In fact, they could amplify the complete part of ITS1, ITS2 and 5.8S rDNA regions and partial part of 18S and 28S rDNA and too many small fragments about 20-30bp. Consequently, using the universal primers, ITSI and ITS4, we created a fragment with variable length about 510- 879 bp of the ITS1-5.8S-ITS2 rDNA region from genomic DNA of several strains of Candida species.(8)

The standard PCR using universal primer pairs ITS1/ITS4 did not produce distinctive bands between C. albicans and C. dubliniensis and thus it is not practical to use them as a tool for species identification. The ITS DNA sequence in NCBI GenBank was clearly different and was used to select restriction enzymes that distinguish the two pathogens in disease diagnosis. PCR-RFLPusing restriction enzyme BlnI produced two fragments with C. dubliniensis and did not digest C. albicans. The PCR-RFLP is a very good tool to distinguish two pathogens (Table- 1).

BlnI makes DNA cleave where there is a CCTAGG sequence. As there is only one cutting site over ITS region of C. dubliniensis, two fragments have been created (200,340 bp). In other hand, since there is no cutting site over ITS region of C. albicans only one fragment that has the same size as PCR has been created.(Table 2)

The restriction patterns generated from ITS regions together with 5.8S rRNA genes have been strongly recommended to display underspecified differences among fungus species.(18)

Table- 1: Distribution of 60 clinical yeast isolates based on types of malignancy.

	Frequency	
Type of Malignancy	**No**	**%**
Brain tumor	**11**	**18.3**
Esophagus cancer	**11**	**18.3**
Acute Lymphoid Leukemia (ALL)	**5**	**8.3**
Breast tumor	**4**	**6.6**
Lymphoma	**5**	**8.3**
Acute Myeloid Leukemia (AML)	**2**	**3.3**
Larynx tumor	**4**	**6.6**
Neck tumor	**2**	**3.3**
Gastric tumor	**3**	**5**
A plastic Anemia	**1**	**1.6**
Lung cancer	**2**	**3.3**
Thyroid cancer	**1**	**1.6**
Liver cancer	**2**	**3.3**
Cheek tumor	**1**	**1.6**
Naso-Pharanx tumor	**2**	**3.3**
Gut tumor	**1**	**1.6**
Tongue tumor	**2**	**3.3**
Prostate tumor	**1**	**1.6**
Total	**60**	**100**

Table 2: Size of ITS1-ITS2 products for C. dubliniensis and C. albicans before and after digestion with BlnI

Candida species	Size of ITS-PCR product	Size of restriction product
C. albicans	**535**	**535**
C. dubliniensis	**535**	**200,335**

Williams et al,(19) evaluated ITS1 and ITS2 regions, together with the entire 5.8S rRNA genes. The sequence variations in the ITS regions were amplified by PCR, using primers ITS1 and ITS4. Although PCR products from both C. dubliniensis and C. albicans had been of similar size (about 540bp), sequence analysis revealed over 20 consistent base differences between the products of the two species. The restriction enzyme MspI yielded two distinct fragments from C. albicans PCR products at the same time as those from C. dubliniensis appeared undigested.(8) The same technique was used by Gee et al,(20) to confirm the existence of two distinct populations within the species C. dubliniensis, designed Cd25 group I and Cd25 group II, respectively, on the basis of DNA fingerprints generated with C. dubliniensis-specific probe Cd25. More recently, Graft et al,(18) established a PCR/RFLP-based system with amplification of regions ITS1 and ITS2 together with the 5.8S rRNA gene, followed by digestion with HpyF10VI, and separation of the DNA fragments on an agarose gel for differentiation of C. dubliniensis from C. albicans.

Based on the present and our previous studies,(9) none of clinical isolate mainly from mucus membrane of patients suffered from cancer identified as C. dubliniensis. Our results are quite consistent with Mirhendi et al.(2) They used the same approach to amplify the ITS region of for discrimination of C. dubliniensis and C. albicans although, their patient populations under study and location of lesions were different. In their study, clinical isolates which mainly originated from skin and nail of patient were suspected to superficial and cutaneous mycoses. Other researchers(21) who have used the same method noted a high prevalence of C. dubliniensis (10.9%) from complete denture wearers. Candida dubliniensis isolates have been primarily recovered from oral and mucosal surfaces, especially in HIV-positive patients. For the first time in Iran(22) Candida dubliniensis isolated (13.3%) from mucus membrane of HIV positive patient but with a different molecular methods.

However, there have been a number of recent reports of its isolation from non-HIV-positive patients.(2, 23) Mokadass et al,(24) reported an overall C. dubliniensis 4.9% among cancer patients by phenotypic and species specific and/or sequencing of ITS regions of rDNA.

However, in this study, we did not identify any C. dubliniensis among all suspected isolates despite of the capability of the method that we used, so further investigations of discrimination between these two morphologically similar species in patients suffering from cancer seem warranted.

Conclusion

In summary, PCR-RFLP of ITS regions with BlnI enzyme is easy and quick to differentiate between C. dubliniensis and C. albicans.

Acknowledgment

We are grateful to the Vice-Chancellor of Research of Mazandaran University of Medical Sciences for financial support.

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Comparison of Prognostic Factors and Death Hazard Function of Acute Myeloid Leukemia (AML) and Acute Lymphoblastic Leukemia (ALL) Patients after Bone Marrow Transplantation

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Abstract

Introduction: The majority of leukemia patients are acute leukemia patients, so that about 70.8% lymphoblastic leukemia were acute lymphoblastic leukemia (ALL) patients and 66.4 % of myeloid leukemia patients were acute myeloid leukemia (AML) in Tehran metropolitan. During the last two decades, intensification of therapy by the use of high-dose Cytarabine allogeneic stem cell transplantation in selected cases, paralleled by improvement in supportive care may have contributed to the impotent. In this article we use parametric survival models for recognizing prognostic factors in acute leukemia patients.

Patients and methods: Data on patients who underwent bone marrow or peripheral blood transplantation were obtained from the Hematology- Oncology and bone marrow transplantation research center at Shariati hospital, Tehran, Iran. Transplantations were performed between Oct. 17, 1993 to Jan. 31, 2007. Written informed consents for hematopoietic cell collection and transplantation were obtained from patients and donors. The study included patients 2 to 56 years of age who had received either an HLA-matched marrow transplant or a marrow transplant with a single HLA mismatch from an unrelated donor. The mean follow- up period was about 2 years after transplantation.

Results: Five hundred and seven patients were included in the study. There were 301 with acute myeloid leukemia (AML) and 206 with acute lymphoblastic leukemia (ALL). The median ages of the AML and ALL patients were 27 (2-55) and 20 years (2-52), respectively. In ALL patients, Prior viral exposure- cytomegalovirus antibody was positive in 143 patients and negative in 30 patients. In AML patients' Prior viral exposure- cytomegalovirus antibody was positive in 220 patients and negative in 41patients. Table- 1 shows the characteristics of 507 patients who included in the study.

Conclusion: In spite of no significant difference in follow-up time, serological status (CMV), donor-recipients sex match, bone marrow cell dose(WBC, CD34, MNC), donor type, source of stem cell, graft type, and conditioning regimen, (Busulfan- Oral, Cyclophosphamide, ALG/AIS/ATG, Stoposide)(Table- 1) in both AML and ALL patients, generalized gamma distribution shows that the mean of SBMT in AML patients is 2.52 times of ALL patients.

Keywords: AML, ALL, Prognostic Factor, Death Hazard Function

Introduction

The majority of leukemia patients are acute leukemia patients, so that about 70.8% lymphoblastic leukemia were acute lymphoblastic leukemia(ALL) patients and 66.4% of myeloid leukemia patients were acute myeloid leukemia (AML) in Tehran metropolitan.(1)

During the last two decades, intensification of therapy by the use of high-dose Cytarabine allogeneic stem cell transplantation in selected cases, paralleled by improvement in supportive care may have contributed to the impotent.(2)

Historically, the prognosis of acute leukemia was based on morphology and cytochemistry. Several factors are known to predict the long-term survival of acute leukemia, including age, cytogenetic, leukocyte count at presentation, previous hematologic disease and prior exposure to

chemotherapy.(3) Identification of prognostic factors related to survival time in patients after bone marrow transplant is very important because we can understand that changing which factors have affect the patients survival time, so it helps a physician making the best decision about patients treatment.

Prognostic factors of acute leukemia were considered by using non-parametric survival methods such as life table, Kaplan-Meier and Cox proportional hazard in many studies.(4, 5, 6, 7) However, the Cox proportional hazard regression model is used extensively, when proportional hazard assumptions are not met, using the Cox proportional hazard model is wrong. Parametric models are attractive because standard method such as maximum likelihood is available for parameter estimation and testing, and proportional hazard assumption is not required.(8)

If the survival time has a specific statistical distribution, the statistical power of parametric survival models is higher than non-parametric survival models.

A parametric survival model is one in which survival time (the outcome) is assumed to follow a known distribution. Examples of distributions that are commonly used for survival time are: the Weibull, the exponential, the Log-Logistic, the lognormal and the generalized gamma.(9) Survival estimates obtained from parametric survival models typically yield plots more consistent with a theoretical survival curve. If an investigator is comfortable with the underlying distribution assumption, the parameters can be estimated in such away that completely specify the survival and hazard functions. This simplicity and completeness are the main appeals of using a parametric approach.(9) Thus, in this article we use parametric survival models for recognizing prognostic factors in acute leukemia patients.

Patients and methods

Data on patients who underwent bone marrow or peripheral blood transplantation were obtained from the Hematology- Oncology and bone marrow transplantation research center at Shariati hospital, Tehran, Iran. Transplantations were performed between Oct 17, 1993 to Jan 31, 2007. Written informed consents for hematopoietic cell collection and transplantation were obtained from patients and donors.

All patients received a BuCy regimen (busulfan 4 mg/kg/day orally on days -6 to -3 and cyclophosphamide 60 mg/kg/day by intravenous infusion on days -2 to -1) for conditioning therapy with subsequent infusion of donor marrow cells on day 0 for GVHD prophelaxy in ALL patients.

All patients were monitored prospectively for the occurrence of adverse events, including GVHD, regimen-related toxicities. Two patients who received cord blood transplants were excluded from the study. 507 eligible patients were enrolled in the study (206 patients were diagnosed with acute Myeloid leukemia and 301 were diagnosed as having acute lymphoblastic leukemia).

The study included patients 2 to 56 years of age who had received either an HLA-matched marrow transplant or a marrow transplant with a single HLA mismatch from an unrelated donor. The mean follow- up period was about 2 years after transplantation.

End points: Platelet recovery was defined by a count of at least 20,000 platelets per cubic millimeters, unsupported by transfusion, for seven days: Neutrophil recovery was defined by an absolute neutrophil count of at least 500 cells per cubic millimeter on three consecutive days. The incidence of acute GVHD was determined in all patients. Patients who developed grades I to IV GVHD, were considered as having AGVHD.(10) The incidence of chronic GVHD was determined in patients who survived for at least 90 days.(11)

Relapse was defined as a recurrence of leukemia. Survival time after bone marrow transplant (STABMT) was defined as the time-interval between bone marrow transplantation and death or censoring. Censoring was defined as being alive at the last follow-up.

Statistical analysis: The probability of STABMT was estimated by using Kaplan-Meier estimator.(12) The probabilities of neutrophil, platelet recovery, AGVHD, CGVHD, death and relapse were calculated with the use of cumulative-incidence-function methods.(12) Confidence intervals were calculated by the use of Log failure transformation. The accelerated time (AFT) models such as: the Weibull, the exponential, the Log-Logistic, the lognormal and the Generalized Gamma distributions were used for finding the distribution of time-to-event (death) after bone marrow transplantation. Discrimination among the Weibull, the exponential, the Log-Logistic, the lognormal the Generalized Gamma distributions and Cox proportional hazard model were done using Maximum likelihood(LL), Akaike criteria (AIC), Cox-snell residuals and graphical methods. By adding different covariates in models,

conditional distributions of parametric and non-parametric survival time models were estimated. The Generalized Gamma distribution was used to determine prognostic factors for survival after bone marrow transplantation. Cox, proportional hazards regression analysis was used when convergence in Generalized Gamma distribution was in question.

In Cox proportional hazards regression models, multivariate models were built using a stepwise forward selection, with a P value of .05 or less considered to indicate statistical significance. Proportional hazards assumption was checked using graphical method, a goodness-of-fit testing procedure [the test of Harrel and Lee (1986)] and the procedure of using time-dependent variables.(9) Smoothed hazard function was estimated using Kernel smoothing method (Ramlu-Hansen 1983).(13) A P-value<0.05 was considered statistically significant. Analyses were completed using SAS ver. 9.1 and SPSS ver. 16 and stata ver. 10.

Results

Five hundred and seven patients were included in the study. There were 301 with acute myeloid leukemia (AML) and 206 with acute lymphoblastic leukemia (ALL). The median ages of the AML and ALL patients were 27 (2-55) and 20 years (2-52), respectively. In ALL patients, Prior viral exposure-cytomegalovirus antibody was positive in 143 patients and negative in 30 patients. In AML patients' Prior viral exposure- cytomegalovirus antibody was positive in 220 patients and negative in 41patients. Table- 1 shows the characteristics of 507 patients who included in the study. The 5-year survival rate based on Kaplan-Meir curve in ALL and AML patients were 52% (95% CI: 47.3-56.7), 65% (95% CI: 60.7-69.3), respectively (Figure- 1). The five-year survival rate in ALL and AML patients in CR1 disease stage was 65% (95% CI: 60.1- 69.9) and 84% (95% CI: 81.3- 86.7), respectively. The shape of hazard function in ALL patients showed that the hazard function had a decreasing trend so that, hazard of dying in the first 6 months after transplantation was higher than, the second six months after transplantation (Figure- 2). The hazard function in AML patients is U- shaped in a way that it decreases till about two years after transplant and then increases till 3 years (Figure- 3).

Prognosis factors of survival after bone marrow transplants, univariate analysis: A number of demographics and transplant-related factors were evaluated using the Gamma distribution as potential risk factors for SABMT. There was statistical deference between SABMT in AML and ALL patients [P=.000, EXP(b) =2.52]; so that, SABMT in AML patients was 2.52 times longer than ALL patients. There were just statistically significant associations among donor age, WBC, CD3, relapse, AGVHD, CGVHD and platelet recovery with SABMT in univariate analysis (Table- 2). There was not any statistically significant association among other variables with SABMT. Every 1000-unit increase in WBC dose cell will increase survival time by 6%. No significant association was observed between SABMT and WBC dose cell in AML patients (P= .18, Exp(b)= 1.1) but in ALL patients this association was considered significant (P= .047, Exp(b)= 1.03). There was a significant association between CD3 dose cell and SABMT (P=.0001, Exp(b)=1.046 CI 95% (1.037, 1.55). Exp(b)= 1.046 shows that with increasing every unit to CD3 cell dose, patients survival time increase about 4.6%.

Three patients in AML group were HLA-mismatched-sibling. There was a significant association between donor type and SABMT (P=.000, Exp (b) =20.45, CI 95% (10.5, 39.2), SABMT of HLA-identical-sibling patients was 19.45 times longer than, HLA-mismatched-sibling patients. The rate of relapse in AML and ALL patients were 16.9% and 28.6%, respectively. Figure- 4 shows cumulative relapse incidence among AML and ALL patients. There was a strong correlation between SABMT and leukemia recurred after transplantation in both ALL and AML patients (p=.000, Table- 2). The patients who had relapsed following transplantation the SABMT of them was about 11.5 time shorter than other patients. The effects of relapse on survival time were similar among patients with ALL and AML. Acute GVHD of grade 1, 2, 3, or 4 developed in 136 patients (77.7%) of ALL group and of 175 (71.8%) of AML patients. The mean of AGVHD time was 13.3 (sd= 16.5) and 15.16 (sd= 14.1) days in ALL and AML patients, respectively. There was no significant difference in the mean time of AGVHD in ALL and AML patients (P= .28). ADVHD have had a significant effect on SABMT in ALL patients (P= .021, Exp(b)= 2.29 CI 95% (1.13, 4.71), but in AML patients its effect was not significant on SABMT (P= .11 Exp(b)= .59 CI 95% (.30, 1.13). The occurrence of AGVHD had a negative effect on SABMT in AML patients, whereas it had a positive effect on SABMT in ALL counterparts. The cumulative incidence of AGVHD after bone marrow among ALL and AML patients has been shown in Figures- 5, 6.

Table- 1. Patients and transplants characteristics.

Characteristics	ALL (n=301)	AML(206)	P-value
Sex, No. (%)			.000
Male	139(67.5)	165(54.8)	
Female	67(32.5)	136(42.2)	
Age mean (sd)	22.5(8.73)	27.4(11.64)	.000
Age group, No. (%)			.000
<15 yr	37(18)	49(16.3)	
16-20 yr	72(35)	55(18.3)	
21-30 yr	64(31.1)	76(25.2)	
31-40 yr	22(10.7)	72(23.9)	
>40 yr	11(5.3)	49(16.3)	
Disease status, No. (%)			.000
CR1	138(67)	218(74.7)	
>CR1	52(25.2)	57(19.5)	
PIF	5(2.4)	9(3.1)	
Relapse 1,2,3,other	11(5.3)	8(2.7)	
FAB or immunophenotype classification, No. (%)	B-lineage: 88(42.7) L1:1(.5) Mature B-cell(L3):4(1.9) Other,specify:93(45.1) T-lineage:6(2.9) Unspecified:14(6.8)	M0:4(1.3) M1:18(6) M2:131(43.5) M3:23(7.6) M4:78(25.2) M5:31(10.3) M6:7(2.3) Other,specify:3(1) Unspecified:3(1)	
Conditioning regimen, No. (%)	185(89.8)	247 (83.2)	.024
BuCy	0	8(2.7)	.04
BuFluATG	204(99)	294(99)	.67
Busulfan - Oral	4(1.9)	2(.7)	.19
Stoposide	186(90.3)	250(84.2)	.031
Cyclophosphamide	4(3.7)	11(3.7)	.192
ALG/AIS/ATG			
Donor age (years), median (range)	21(2-55)	25(1-54)	
Patients age (years), median(range)	20(2-51)	27(2-55)	
Donor sex, No. (%)			
Female	85(41.3)	112(37.2)	
Male	121(58.7)	189(62.8)	
Graft type, No. (%)			.175
Allogeniec	200(97.1)	297(98.7)	
Syngeneic	6(2.9)	6(2.9)	
Source of stem cells, No. (%)			.43
Bone marrow	14(6.8)	23(7.8)	
Peripheral blood	192(93.2)	278(92.4)	
Donor type, No. (%)			.35
HLA-identical sibling	198(99)	293(99.7)	
HLA-mismatch - sibling	2(1)	1(.3)	
Bone marrow cell dose, median (range)			
WBC	10.5(2.1-33.3)	10.32(2.1-24.5)	.12
CD3	29(1-81.1)	25(.2-74.6)	.46
$CD34^{+}$cells($\times 10^{6}$/kg)	2.15(.2-18.6)	1.9(.2-79.2)	.42
MNC	6.44(.94-19.93)	6.95(1.04-17.6)	.22
Donor– recipient sex match，No. (%)			.19
Male-male	85(41.3)	106(35.2)	
Male-female	54(26.2)	59(19.6)	
Female-male	36(17.5)	83(27.6)	
Female-female	31(15)	53(17.6)	
Donor- Negative-negative recipient serological status For cytomegalovirus, No. (%)			.96
Negative-negative	16(9.3)	24(9.4)	
Negative-positive	19(11)	32(12.5)	
Positive-negative	14(8.1)	17(6.7)	
Positive-positive	123(71.5)	182(71.4)	

Outcomes, No. (%)			
Death	76(39.9)	67(22.3)	.000
Relapse	59(28.6)	51(16.9)	.002
AGVHD	136(77.7)	188(71.8)	.136
CGVHD	34(24.1)	62(25.5)	.76
Platelet recovery	141(75)	224(80)	.175
AGVHD time			
Mean(sd)	13.3(16.5)	15.16(14.12)	.28
Median	9	10	.003
Range	3-90	2-90	
CGVHD time			
Mean(sd)	160.26(73.4)	181.80(83.1)	.25
Median	140	168	.15
Range	91-327	91-492	
Relapse time			
Mean(sd)	580(555)	703.2(645)	.056
Median	412	479	.036
Range	10-2661	21-4301	
Platelet recovery time			
Mean(sd)	20.8(19.2)	19.2(10.1)	.32
Median	17	17	.80
Range	1-165	2-90.8	
Neutrophil recovery time			
Mean(sd)			
Median			
Range			
Follow up-month			.30
Median	16	17	
range	3-89	3-143	

Among patients who survived for 90 days or longer, chronic GVHD developed in 62 (25.5%) AML and 34 (24.1%) ALL patients. The median time of CGVHD was 123 (SD=74.1) and 156 (SD=84.9) days in ALL and AML patients, respectively. There was a significant association between CGVHD and SABMT in both AML and ALL patients (P=.000), so that in the acute leukemia patients with CGVHD, survival time was about 3.11 times longer than the patients without CGVHD (Figures-.7, 8). The cumulative incidence of CGVHD after transplantation in AML and ALL patients has been shown in Figure- 9.

Among patients who had platelet recovery, the mean time was 19.88 days, CI 95% (18.4- 21.36). There was strong association between platelet recovery and SABMT in acute leukemia patients [P= .000, Exp(b)= 3.11, CI 95% (1.95-4.95) Table-2, Figures- 10, 11]. The STABMT in ALL patients who had platelet recovery was 3.39 time longer than the patients how did not have platelet recovery. The cumulative incidence of platelet recovery in ALL and AML patients has been shown in Figure-12.

Prognostic factors of survival after bone marrow transplants, multivariate analysis: The variables that showed a significance level of P-value<.2 on univariate analysis were considered in the multivariate models, also the patients age and sex were considered in the variable selection process. Several multivariate models were considered because of strong association between SABMT and relapse and avoiding of missing data in cd3 and WBC variables. Discrimination among exponential, Weibull, log-normal, log- logistic and gamma distributions was done by likelihood ratio test.(14) The three-parameter generalized gamma distribution was shown to be appropriate for data set. All models were estimated using Generalized Gamma distribution. When the assumption of the Cox proportional hazard regression model was met and generalized gamma distribution was known to have convergence problem we used proportional hazard regression model in our study.

Death risk adjusted for patients sex and age in ALL group was 2.14 times of AML counterparts (All vs. AML, Hazard ratio (HR)= 2.14 CI 95% (1.52-3.10) P=.000). Other models were estimated in AML and ALL patients, separately. In ALL patients, a strong association between relapse adjusted for patients' sex and age and SABMT (exp(b)= 10 CI 95% (5.2-19.3) p= .000) showed that SABMT in the patients who did not have relapse was about 10 times longer than the patients with relapsed disease. CGVHD adjusted for patients' sex and age had significant association with SABMT (exp (b) =5.4 CI 95 % (2.57-10.38) P=.0001).

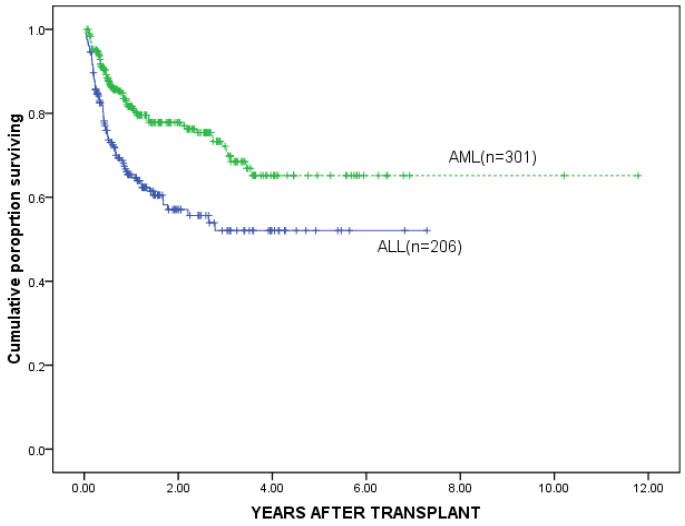

Figure- 1. Kaplan-Meir estimated survival after transportation for patients diagnosed with AML compared with ALL. Differences did reach statistical significance as determined by the log-rank test (p=.000)

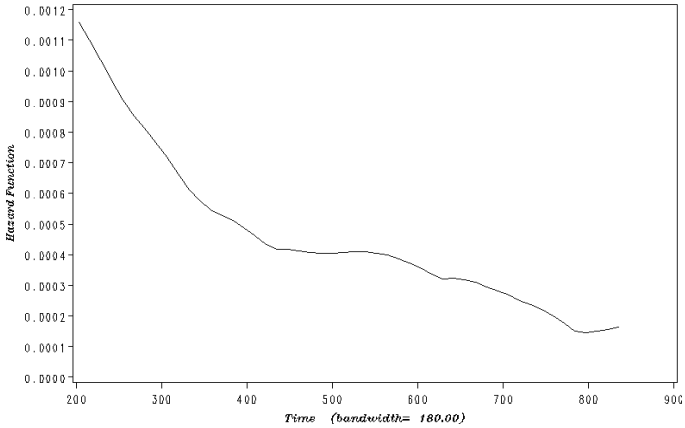

Figure- 2. Hazard function in ALL patients.

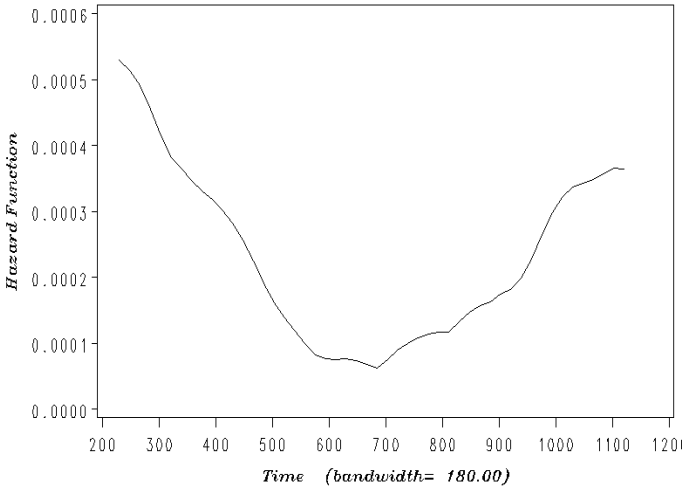

Figure- 3. The hazard function in AML patients.

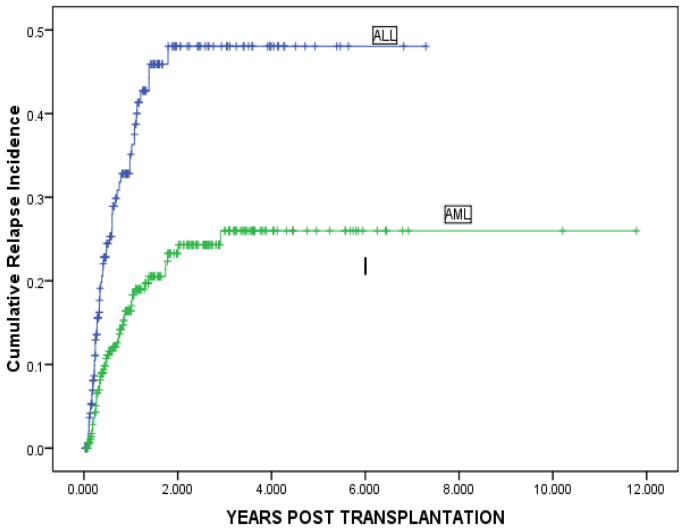

Figure- 4. Cumulative relapse incidence after transportation for patients diagnosed with AML compared with ALL. Differences did reach statistical significance as determined by the log-rank test (p=.000).

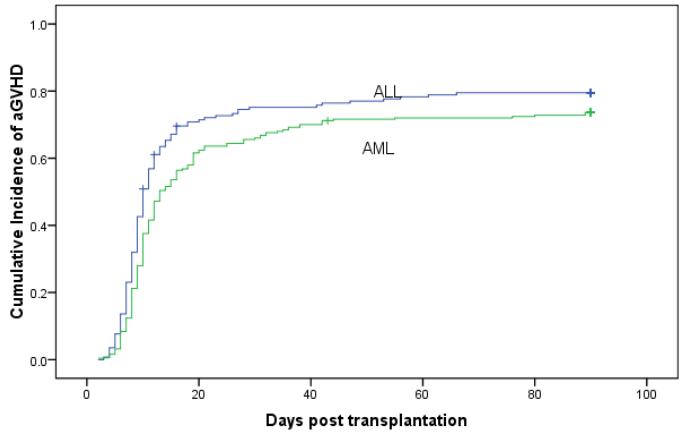

Figure- 5. Cumulative Incidence of aGVHD for patients diagnosis with AML compared with ALL.

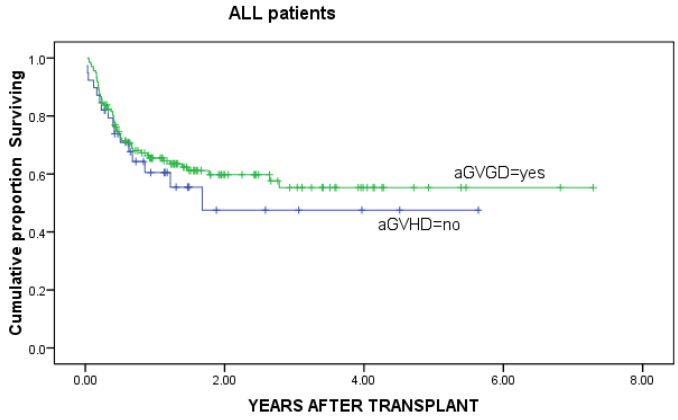

Figure- 6. Survival of ALL patients after transplantation grouped according to aGVHD development.

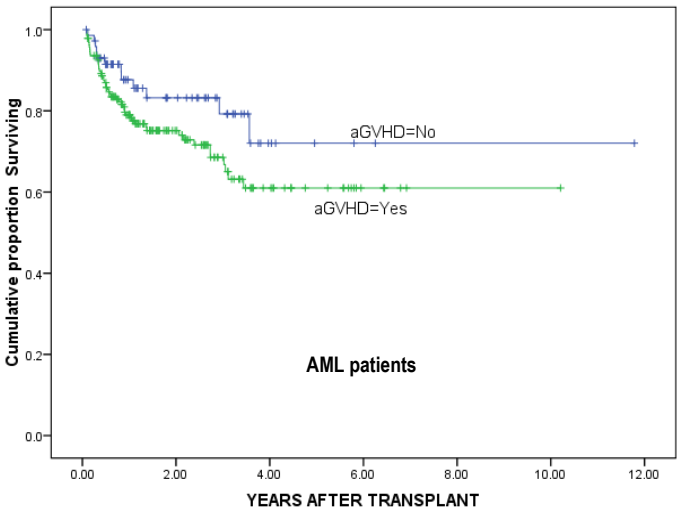

Figure-7. Survival of AML patients after transplantation grouped according to aGVHD development.

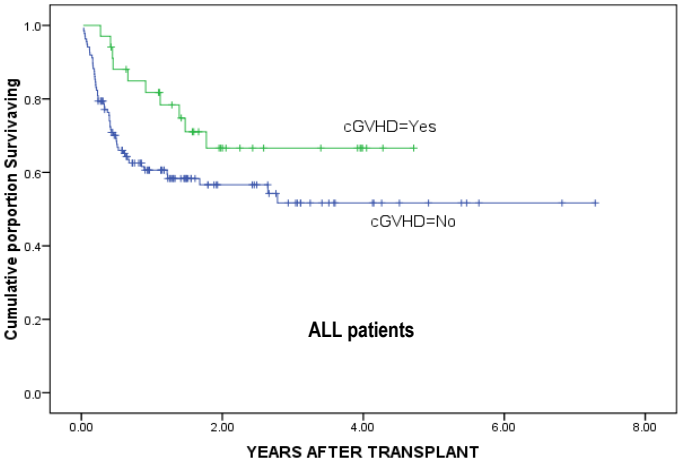

Figure- 8. Survival of ALL patients after transplantation grouped according to cGVHD development.

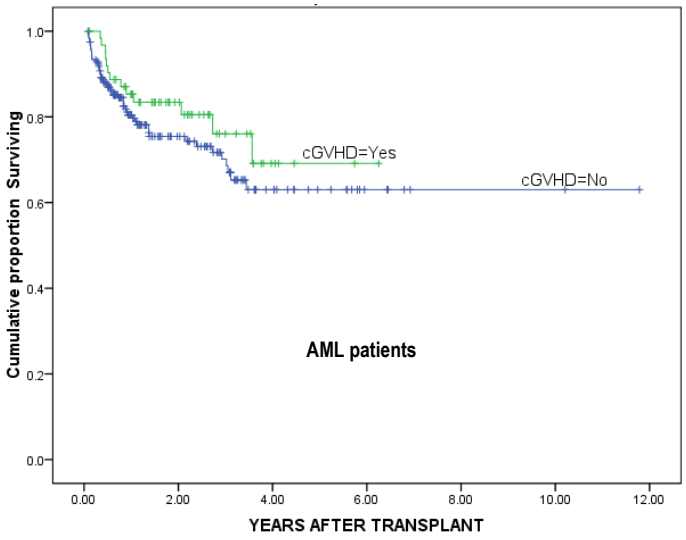

Figure- 9. Survival of AML patients after transplantation grouped according to aGVHD development.

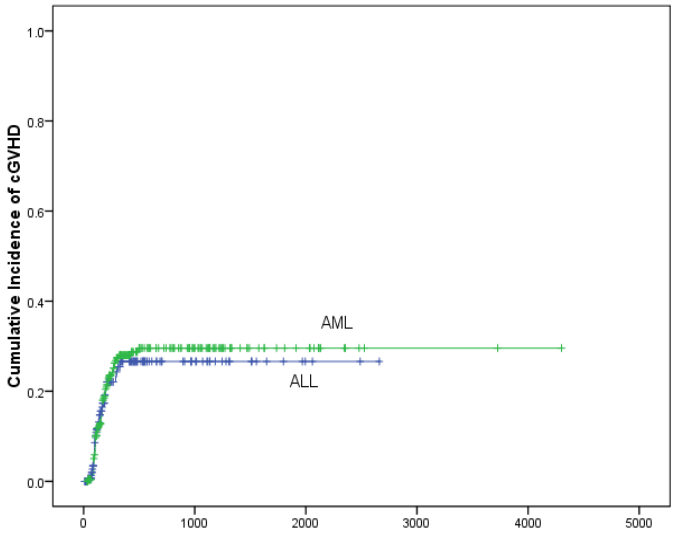

Figure- 10. Cumulative Incidence of platelet recovery.

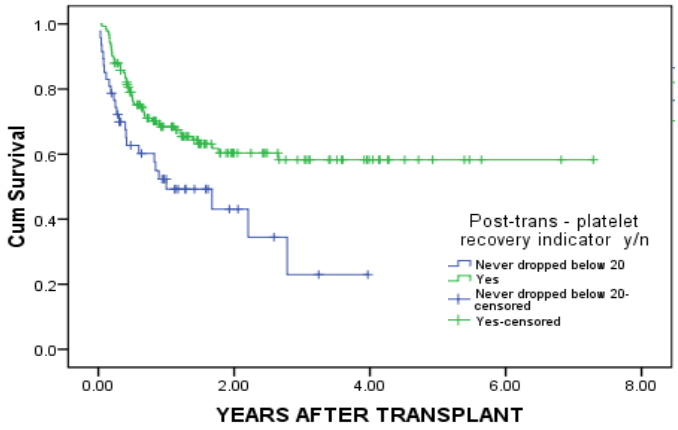

Figure- 11. Survival of ALL patients after transplantation grouped according

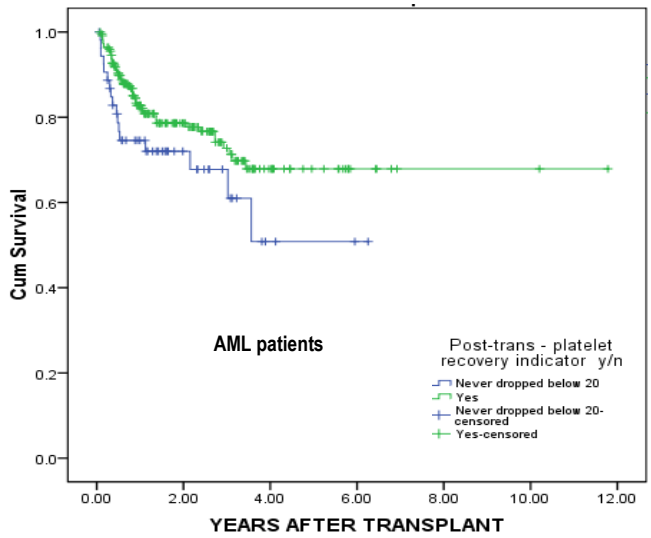

Figure- 12. Survival of AML patients after transplantation grouped according to platelet recovery.

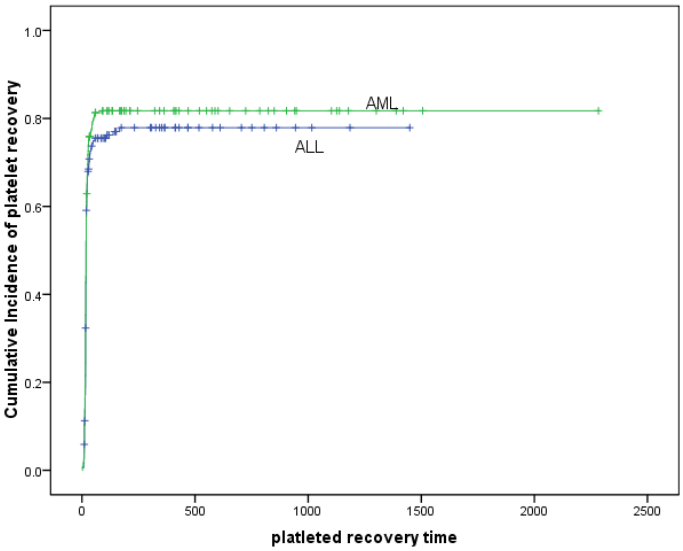

Figure- 13. Cumulative Incidence of platelet recovery.

Platelet recovery adjusted for patients' sex and age had significant association with SABMT (exp (b)= 3.7 CI 95 %(2.34-5.68) P=.0001). Because of convergence problems we could not use GG distribution for considering the relation of AGVHD adjusted for patients' sex and age. Cox proportional hazard regression model showed there was no association between AGVHD and SABMT adjusted for patients' sex and age (AGVHD no vr yes HR=1.01 CI 95 % (.98-1.039). CGVHD adjusted for AGVHD and Platelet recovery had significant association with SABMT (exp (b) =3.6 CI 95 % (1.95-6.8 P=.0001). Platelet recovery adjusted for AGVHD and CGVHD had significant association with SABMT (exp(b)= 3.3 CI 95% (1.61-6.7 P=.001). There was no significant association between WBC dose cell adjusted for patients' sex and age and SABMT (exp(b)= 1.02 CI 95% (.92-1.13) P= .65).

In AML patients, in a model including relapse, patients sex and age, there was strong association between relapse and SABMT (exp(b)=10.58, CI 95% (5.4-20.7) P= .000) In this model, the significant association between patients' sex and SABMT(exp(b)= 2.05 male vr. Female CI 95 % (1.1-3.7) (P=.000), showed that SABMT in males was about two times of females. There was no significant association between SABMT and patients age (Exp (b) =.97 CI 95% (.95-1.01) P=.098).

In AML patients, significant association between WBC dose cell adjusted for patients' sex and age and SABMT (Exp (b)= 1.13, CI 95 % (1.037-1.23), P=.005) showed that with increasing every unit in WBC dose cell, SABMT of patients increased about 13%, CI 95% (3.7% to 23%). Because of convergence problems in the generalized gamma model, Cox proportional- hazard regression model was used to consider the relationship among AGVHD, CGVHD, patients' sex and age and platelet recovery in a multivariate model. In this model the significant association between platelet recovery and SABMT (HR=2.42, CI 95% (1.3-4.45) P=.004) showed that death risk in patients with no platelet recovery is 2.42 times of those who had platelet recovery. AGVHD effect adjusted for platelet recovery, CGVHD, patients' age and sex had significant association with SABMT (HR=.47 CI 95 % (.24- .93) P=.03) which showed that death risk in AML patients who developed AGVHD was about 2.12 (1/.47) of the patients without developing AGVHD.

Discussion

Our objectives were to find hazard function shape in AML and ALL patients with HLA-matched bone marrow and to identify prognostic factors of SABMT using parametric and non-parametric model. Risk of death after bone marrow transplantation had different patterns in ALL and AML patients so that hazard function had a decreasing rate in ALL patients, that is it decreased after transplant until about 2 years and then increased until 3 years after transplant .The reasons of difference in shape of hazard function in ALL and AML patients were not clear but it seems that relapse of disease after two years leads to increase in the hazard of death in AML patients. The Generalized gamma distribution provides better fit than other parametric survival models such as: exponential, Weibull, log-normal, log- logistic. Hazard function in the Generalized gamma distribution can take a wide variety of shapes.(13) When I executed PubMed search, I could not find any research regarding generalized gamma distribution for finding hazard shape after bone marrow transplant or finding prognosis factors of SABMT in ALL and AML patients. In many researches Log- rank test or Cox proportional-hazard regression models were used to consider prognostic factor's of SABMT.(4- 7, 15, 16)

In our study the five-year survival rate based on Kaplan-Meir curve in ALL and AML patients with sibling donors in CR1 disease stage was 65% CI 95% (60.1 -69.9%) and 84% CI 95%(81.3 -86.7%), respectively Based on Kaplan-Meir curves of data from the center for international blood1 and marrow transplant research (CIBMTR) and the national marrow donor program (NMPD) data the rates were 65% and 65% ,respectively.(17) The five-year survival rate in AML and ALL patients is ranging from 11% for patients over 55 years to 71% in

infants and children.(18- 22) The five- year survival rate was only 5% CI 95% (1-15%) among patients who were not transplanted in first remission.(20) Thus, improvement in patients survival seems to be associated with the increasing use of transplant.

In spite of no significant difference in follow-up time, serological status (CMV), donor-recipients sex match, bone marrow cell dose (WBC, CD34, MNC), donor type, source of stem cell, graft type, and conditioning regimen, (Busulfan- Oral, Cyclophosphamide, ALG/AIS/ATG, Stoposide) (Table- 1) in both AML and ALL patients, generalized gamma distribution shows that the mean of SBMT in AML patients is 2.52 times of ALL patients.

Cox proportional- hazard regression models show that death hazard in ALL patients is 2.14 times of (CI 95 %) AML patients.

One of the reasons that the survival of AML patients is better than ALL patients is the higher relapse rate in ALL group.

The rate of relapse in AML patients was 16.9%, whereas it was 28.6% in ALL counterparts.

In this study CGVHD developed in 24.1 % of ALL patients and in 25.5% of AML patients.

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Serum Galactomannan and Diagnosis of Invasive Aspergillosis in Patients with Hematologic Malignancies, Blood and Marrow Transplantation

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Abstract

Introduction: Invasive aspergillosis is a major cause of morbidity and mortality in hematologic malignant patients which have received intensive cytotoxic therapy or undergone blood and marrow transplantation (BMT). Early clinical and radiological diagnosis of IA is almost impossible and so mortality is very high. The main purpose of our study was to assay the diagnostic value of serum galactomannan (a fungal antigen found in the sera of infected patients) level in early diagnosis of invasive aspergillosis in patients with hematologic malignancies and BMT.

Method and materials: During 2009and2010, 70 adult patients with neutropenic fever of unknown origin in Shariati and Imam Khomeini centers of cancer and BMT were tested for galactomannan serum level on the 7th day of fever or before starting antifungal treatment. The OD index of 0.5 or more was taken as positive result. Tissue biopsy from lung or sinus was performed in 8 patients and there were 7 positive results for aspergillus species. Galactomannan EIA test result was positive in 26 cases (37.1%). There were 7 cases of proven IA with positive tissue (lung or sinus) biopsy and 20 cases of probable IA. Overall, 27 cases were positive for IA (gold standard= proven IA+pobable IA).

Results: The study yields a sensitivity and specificity of 77.8% and 88.4% for this test, respectively. Positive and negative predictive value of the test with confidence interval of 95% was 80.8 % (60-92.7%) and 86.4 %(72.0-94.3%), respectively.

Conclusion: Considering these results, galactomannan EIA test results must be interpreted cautiously as an alternative test to prove IA, and it doesn't eliminate the need for other diagnostic criteria such as clinical symptoms, biopsy, imaging, etc. However, the test is substantially helpful in recognizing true cases of the disease.

Keywords: Invasive Aspergillosis, Galactomannan, BMT

Introduction

Invasive Aspergillosis (IA) is a common and seriously lethal infection in immunocompromised patients. The disease is a major cause of morbidity and mortality in hematologic malignant patients which have received severe cytotoxic therapy or undergone blood and marrow transplantation (BMT).(1) The major risk factor is severe prolonged neutropenia. However, deficiency in host immunologic response because of longterm steroid therapy, GVHD and CMV infections are also major risk factors in relapse and progression of Aspergillosis after bone marrow transplantation.(2) Recent epidemiologic studies have shown a 3- 4 time increase in aspergillosis infection prevelance in major cancer centers due to using high dose chemotherapy and newer immunosuppressive agents.(1, 3-7) It's incidence exceeds 30% in some reports.(1) It has been reported that the most common community acquired pathogen in allogenic hematopoietic stem cell transplantation (allo-HSCT) recipients is aspergillus(8) and despite the use of itraconazole and fat based formulations of amphotricin B, mortality of the infection is more than 80%.(3, 9, 10)

Pulmonary Invasive Aspergillois is mainly diagnosed by clinical and radiological findings. "halo sign" is a radiological finding that makes the early diagnosis of IA in neutropenic patients possible but is not sensitive enough (33-60%) nor specific. The other radiological finding is Air Crescent sign which is not found until third week of infection and that is the time which the infection is completely established.(12)

Hence, early clinical and radiological diagnosis of IA is almost impossible, and the mortality is very high (47-92%).(13) To increase survival, early and definite diagnosis of invasive fungal infection is a major challenge.(14, 15) Sampling strategies for tissue culture are hardly done successfully in ill and mainly thrombocytopenic patients and sometimes could not be done at all. On the other side the sensitivity of culture is low and it takes days or even weeks for the positive results to be ready. The other problem is the probability of contamination and colonization which affects the results, so there is a need for creation of new non-cultural methods for early detection of IA before damaging target tissues. Those tests that detect fungal antigens or metabolites released by fungus are hopeful. Development of rapid, reliable, specific and non-invasive test is a major revolution in patients management. One of the new methods for diagnosis of IA is galactomannan EIA test. Galactomannan (GM) is a polysaccharide part released from the hyphea wall during its growth in tissue and is found in serum. Detection of galactomannan in circulation is the important tool for early diagnosis of IA. This antigen is found by common method of Sandwich Elisa (platelia-Aspergillus Elisa).

Studies done by food and drug administration (FDA) to approve the test have reported a sensitivity of 79% in adult patients. Reported sensitivity and specificity of this test is ranged between 30-100% depending on the patient population, diagnostic methods and definitions used for diagnosis of IA.(12, 16-18, 23-27) Sensitivity of the test is dependent on the criteria used to diagnose IA, usage of antifungal regimens as prophylaxis or therapy, use of different cut off points and host immunity states.(19, 21) Because galactomannanemia is transient, perhaps inadequate and inappropriate sampling strategies could affect the sensitivity of the test.(20) Specificity of the GM EIA is ranged between 78-100% based on the used cut off point and also the study population.(28)

Overall, it could be said that GM assay is a very specific test for detecting IA. However, false positive result of the test happens in about 2% of normal population, as reported by Maertens.(29) False positive results are listed as follows: use of fungus derived antibiotics such as amoxicillin-clavulanic acid, piperacillic- tazobactam, ampicillin- sulbactam(20, 21, 30 - 33) children and infants,(34, 35) bacteriemia,(36) dialysis,(37), auto-antibodies(38). COPD and cystic fibrosis undergoing lung transplantation show more false positive results,(39) and at last, false positive results are found more frequently during 2 weeks after receiving cytotoxic drugs in patients with hematological malignancies and 30 days after HSCT.(41)

Considering the high prevalence and mortality of this lethal infection in cancerous patients and lack of proper diagnostic tools in our country, in this study we tried to determine the diagnostic value (sensivity, specifity, negative and positive predictive values) of a sandwich Elisa test for diagnosis of IA (which can detect 0.5 nanogram of galactomannan in one ml of serum in 3 hours, Platelia Aspergillus Immunoenzymatic Assay; Biorad Laboratories, Hercules, CA) in suspected patients in health system of our country.

Materials and Methods

Study population: In Imam Khomeini and Dr Shariati centers of hematology, oncology and Bone marrow transplantation, following patients with fever and neutropenia (OT> 38.3 'c and ANC<500) during therapy, were included in the study during the years of 2009 and 2010:

1- All of the patients with the following diseases whom received chemotherapy and had an expected neutropenic episode at least for 10 days:

- Acute leukemia or its relapse
- Chronic myeloid leukemia (CML) which receive AL like induction regiment.
- Lymphoblastic non Hodgkin's lymphoma
- High risk myelodysplasia [refractory anemia with excessive blasts (RAEB), refractory anemia with excessive blasts in transformation (RAEBt), secondary acute myelogenous leukemia (sAML)]

2- Patients undergoing BMT or HSCT

Patients under 16 years old and those receiving piperacillin-tazobactam or co-amoxiclav were excluded.

Treatment of fever and neutropenia was started by broad spectrum antibiotics according to the 2002 Guidelines for the Use of Antimicrobial Agents in Neutropenic Patients with Cancer .Blood culture and Physical exam were done daily with particular attention to sinusal and pulmonary signs and symptoms. Other diagnostic methods such as CXR

(2-3 times a week during persistent fever), sinusal x-ray, sputum analysis and culture, urine and stool culture were administered.

In those patients that fever persisted for 48-72 hours, a glycopeptide antibiotic was added to therapeutic regiment. CT scan was done in these circumstances:

- Persistence of fever with unknown origin for more than 5 days despite receiving appropriate antibiotics.
- Abnormal findings in CXR or CT scan or
- Manifestation of pulmonary infection symptoms

High resolution computed tomography (HRCT) of thorax was done during full inspiration. According to clinical symptoms and graphy of sinuses, CT scan of paranasal sinuses was ordered and administered. Consultation with an ENT specialist was done and if patient circumstances were stable and suitable, sinus endoscopy and biopsy from lesions were performed. If pulmonary infiltration was present and patient's platelet number was more than 20000 without hypoxia, bronchoalveolar lavage (BAL) and lung biopsy were accomplished. The sample taken from BAL was analyzed and cultured for mycobacterium, bacteria and fungus and acid fast stain was also done. For detection of fungal hyphea invasion, tissue samples were stained by silver or periodic acid shiff (PAS).

Anti fungal treatment (IV Amphotericin B) was started even if one of the following states happened: fever despite 7 days of appropriate IV antibiotic therapy, development of a new infiltration on CXR, characteristic feature of invasive mycosis on CT scan, detection of fungus in pulmonary or paranasal sinuses samples, sudden intracranial lesion compatible with invasive mycosis, relapse of fever in a neutropenic patient after 48 hours without fever while receiving broad spectrum antibiotics.

Sampling and detecting galactomannan: Patients with neutropenic fever were carefully observed for symptoms and signs of IA every day. Before starting antifungal treatment, 5 cc of blood was taken by the nurse to obtain the serum needed for galctomannan EIA test. The samples were stored in -80'c and sent to reference laboratory. The galactomannan antigen was detected by Bio-Rad labratories Platelia Aspergillus EIA, following the instruction suggested by the manufacturing company. The OD index of 0.5 or more was taken as positive result. The results of the test were documented in the questionnaires by investigators.

Definitions: Patients were categorized based on European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) definitions with a little change:

-proven IA: histopathologic document of tissue invasion (sample from needle aspiration, biopsy or autopsy) with filamentous fungi which have septate hyaline hyphea and positive cultures for aspergillus.

-probable IA: positive culture or cytology for aspergillus species from sputum sample or discharges of lower respiratory airways or BAL sample plus a major criteria (halo sign or air crescent sign or cavity inside a consolidation in CT scan) or at least two of these minor criteria: lower respiratory track symptoms such as cough, dyspnea, hemoptysis, pleuretic chest pain ; pleural rub ; every new infiltration which is not compatible with major criteria and has no other diagnosis.

A group consisted of a radiologist, an infectious disease specialist and at least two hematologists along with a medical oncologist, was defined to make the final decision about patient's disease definition based on clinical criteria, EORTC/MSG criteria and response to therapy. Their final decision was made as probable IA. The gold standard was the sum of probable and proven cases.

Statistical analysis: Underlying variables such as age, sex, etc. and results of HRCT, sinus CT scan, BAL, biopsy, clinical diagnosis and test results were obtained from questionnaires for each patient. The value of each variable was put in a code sheet through Excell**.** Finally, the data were statistically analyzed using SPSS 15 software, version 15 and Epi-info version 6 and subsequently, statistical indices and sensitivity, specificity, positive predictive value and negative predictive value of the test were calculated.

Results

70 patients enrolled in our study. There were 41(58.6%) men and 29(41.4%) women. The mean age was 44(16-60). The most frequent diseases were acute myeloid leukemia, ALL and non Hodgkin's lymphoma which had a frequency of 27(38.6%), 18(25.7%) and 6(8.6%), respectively. Other diseases were Hodgkin's disease, aplastic anemia, multiple myeloma, talassemia, amyloidosis, and CML, respectively.

33(47%) patients underwent allogenic BMT from their brothers (21) or sisters (12).

21(30%) patients underwent autologus BMT. 16(22.9%) of patients were not cases of BMT.

GVHD was found in 13(18.6%) patients who were totally under treatment with steroid.

HRCT was done for all of the patients and radiologist reported mycosis infection features in 23 patients (31.4 %). Paranasal sinuses CT scan was performed in 33(47%) patients and sinusal involvement was reported in 6 (8.5%) cases.

BAL was done in 8 of patients and the result was positive for aspergillus in 5 cases (7.1%) and the other 3 patients (4.3%) had negative results. Lung biopsy performed in six of these 8 patients (two patients didn't have suitable clinical state for biopsy) revealed positive and negative results for aspergillosis infection in 5 cases and one case, respectively. All of the 5 positive lung biopsies had positive BAL .One patient who had negative lung biopsy was negative for BAL too.

Sinus biopsy was done in two patients and both of them had positive results for aspergilosis smear.Overall, tissue biopsy from lung or sinus was performed in 8 patients and there were 7 positive results in biopsies taken. (Table-1)

Galactomannan EIA test result was positive in 26 cases (37.1%) of all 70 patients.

Cases of proven and probable IA were defined according to the definitions mentioned above. There were 7 cases of proven IA with positive tissue (lung or sinus) biopsy and 20 cases of probable IA. So overall, positive cases of IA (gold standard= proven IA+pobable IA) were 27. The galactomannan EIA test results were compared to these results. All of the cases with positive biopsy results had positive results in galactomannan EIA too. The patient with negative biopsy and BAL result for aspergillus had negative result in galactomannan EIA test too. Diagnostic value of galactomannan EIA test in diagnosis of invasive fungal infection was compared with HRCT results: 38 patients were negative for both CT findings and GM test result. 10 patients with negative CT scanning for aspergillosis had positive GM test. There were positive results for both CT findings and GM test in 16 patients.finally ,6 patients with positive CT scanning for aspergillosis had negative GM test.(Table- 2)

Diagnostic value of galactomannan EIA test in diagnosis of invasive fungal infection was compared with gold standard test and the results were as follow:

Sensitivity of the test with confidence interval of 95% was 77.8% (57.3-90.6%).

Specificity of the test with confidence interval of 95% was 88.4% (74.1-95.6%).

Positive predictive value of the test with confidence interval of 95% was 80.8 % (60-92.7%).

Negative predictive value of the test with confidence interval of 95% was 86.4% (72.0-94.3%).

Table 1. Tissue biopsy and GM test results

		Tissue Biopsy (proven)	
GM test results Test		Negative	Positive
	Negative	1 (lung)	0
	positive	0	7 (5 lung)

Table 2. HRCT and GM test results

	HRCT		
Test		negative	positive
	Negative	38	6
	positive	10	16

Table 3. Diagnostic value of test by HRCT resutls		
		95% Confidence
sensitivity	72.7	49.6-88.4
specifiity	79.2	64.6-89.0
Positive predictive value	61.5	40.7-79.1
Negative predictive value	86.4	72.0-94.3

Table 4. Diagnostic value of test by gold standard resutls		
		95% Confidence
sensitivity	77.8	57.3-90.6
specifiity	88.4	74.1-95.6
Positive predictive value	80.8	60.0-92.7
Negative predictive	86.4	72.0-94.3

Discussion

In our study galactomannan testing yield sensitivity of 77.8% and specificity of 88.4% for detection of invasive aspergilosis. Previous studies done so far, have reported a sensitivity between 30-100% and specificity of 78-100% for this test. Our results are nearly compatible with the results of Pfeiffer meta-analysis on 27 studies on this subject between 1966-2005 which reported a sensitivity of 71% and specificity of 89%. In some studies, a positive test was accepted if the retest resulted in positive too. Since, the test was relatively expensive and our patients had poor economic condition we couldn't retest the positive results except in a few cases. Galactomannan EIA test has not been supported by health insurance systems in our country yet. Newsworthy, we wanted to evaluate the diagnostic value of the test in status quo and real economic conditions of our patients. Surprisingly, one time testing also had approximately the same results as meta- analysis. However, because of the small

number of our study population, this finding could be considered accidental.

Since the sensitivity of the test is not high enough, therefore it can not be used as a screening test at least in situations similar to our study (detecting galactomannan on 7th days of neutropenic fever). Perhaps it is needed to find a group of high- risk patients in whom the sensitivity is higher.

Considering these issues, it is concluded that galactomannan EIA test results must be interpreted cautiously as an alternative test to prove IA, and it doesn't eliminate the need for other diagnostic criteria such as clinical symptoms, imaging, and biopsy. However, the test is substantially helpful in recognizing true cases of the disease (specificity of 88.4%). The high negative predictive value of the test (86.4%) represents that a negative result for galactomannan EIA can strongly decrease the probability of IA. The positive predictive value of 80.4% suggests that a positive result of this test can acceptably demonstrate the probability of the disease.

The single brand of the kit (Bio-Rad labratories Platelia Aspergillus EIA) is a positive point in the evaluation of the test in this study.

One problem in our study and almost every other study done in this setting is the lack of enough positive cases proven by biopsy as the gold standard test, because biopsy can not be performed for the majority of cases due to thrombocytopenia and their unstable conditions. It is remarkable that in this study all 7 patients with positive biopsy for aspergillus had positive results in galactomannan EIA test and the only negative biopsy had negative result in Galactomannan assay. This is valuable, however, it may be not reliable and judicable because of the small number of patients in whom biopsy was taken.

It is suggested that further studies should be done with attention to early diagnosis of IA by using galactomannan EIA test before day 7, effects of antifungal therapy on test value and the relation between serum galactomannan level and patients outcome.

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Bi-Atrial Primary Cardiac Lymphoma: A Rare Entity

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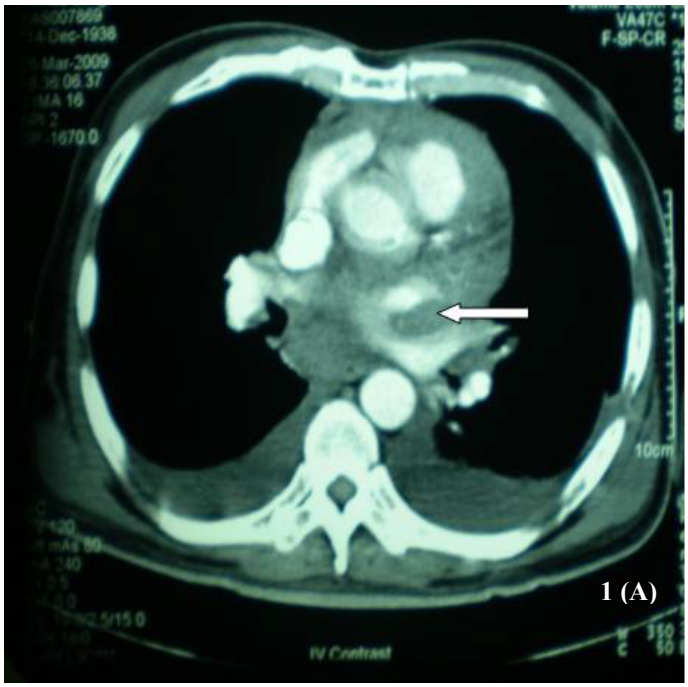
Abstract
Primary cardiac lymphoma is a rare disease which mainly found in elderly men. It is usually a B-Cell non-Hodgkin's lymphoma which primarily located in the heart and may involve the pericardium. The common presentations include massive pericardial effusion and heart failure. Clinical diagnosis is often delayed in these patients and prognosis is dismal. We report a case of a 70-year-old man presented with congestive cardiac failure and constitutional symptoms. A computed tomography of the chest showed two large right and left atrial masses. Echocardiographic study demonstrated that the tumour was in both atria with infiltration into the left ventricle. The diagnosis was confirmed by lymph node excision. The patient was started on chemotherapeutic agents but unfortunately succumbed to the disease 18 days later. Although, the prognosis of primary cardiac lymphoma remains poor, early diagnosis may alter the clinical course.

Keywords: Primary Cardiac Lymphoma, Tumour, B-Cell, Echocardiography, Histopathology

Introduction
Primary cardiac lymphoma is defined as lymphoma involving only the heart or the pericardium, or when the main bulk of disease is localized to the heart. Primary cardiac lymphoma is extremely rare with only 77 cases reported up to 2007. (1) The reported incidence is about 0.5% of all lymphomas and about 1-2% of all cardiac tumours. (2-6) Clinical diagnosis is very difficult and often become apparent when the disease is advanced, with the majority of cases identified at postmortem. (5) Patients may present with signs of heart failure, arrhythmias, or massive pericardial effusion depending on the location of the tumour. The usual sites of involvement are right atrium, right ventricle, pericardium, left ventricle, interventricular septum and left atrium in the descending order of frequency. (7) In the present case, both right and left atria were affected by primary lymphoma. Similar findings have only been reported in three patients. (7)

Case Report
A 70-year- old immunocompetent man was admitted to the hospital and was found to be suffering from congestive cardiac failure. He also suffered from anorexia, weight loss and nocturnal sweating prior to admission. There had not been any further manifestation of the disease until two months before presentation. The patient who was an ex-smoker had a history of diabetes mellitus and hypertension for the past 10 years which were controlled with oral hypoglycemic agents and anti-hypertensive. The patient had an unremarkable family history.

Physical examination revealed a cachexic man with tachypnea and jugular vein distention. On physical examination the patient was pale but not jaundice. The blood pressure and pulse rate were normal. A soft systolic murmur grade 2/6 was heard at the lower left sternal edge with an audible third heart sound. The breath sounds were reduced at the lung bases and fine crackles with occasional rhonchi were audible.



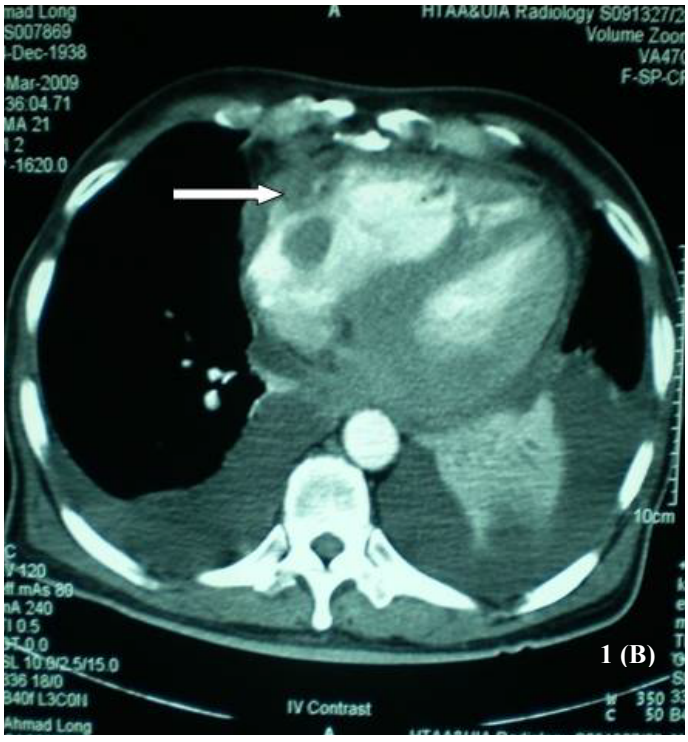
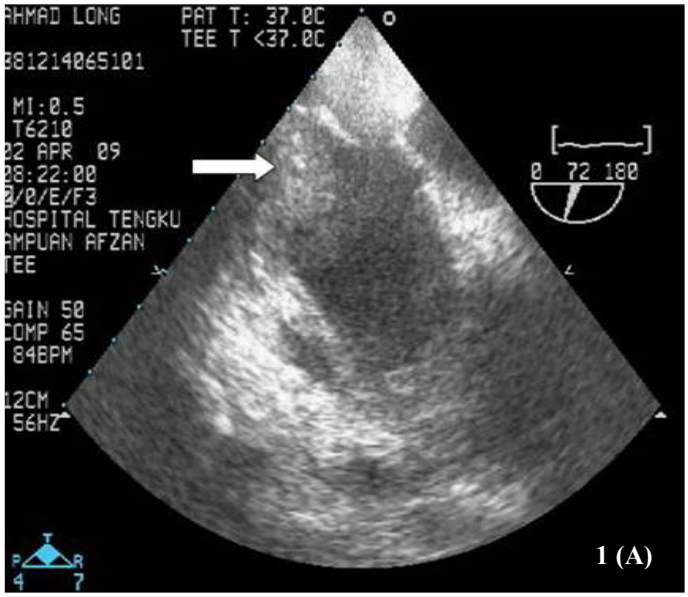


Figure- 1 (A and B): These figures were the thorax computed tomography showed the filling defect in the left and right atrium which was the lymphomatous mass.



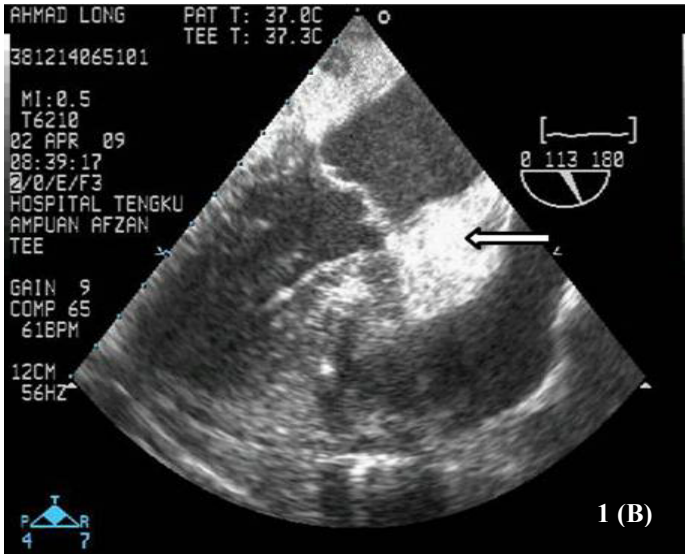
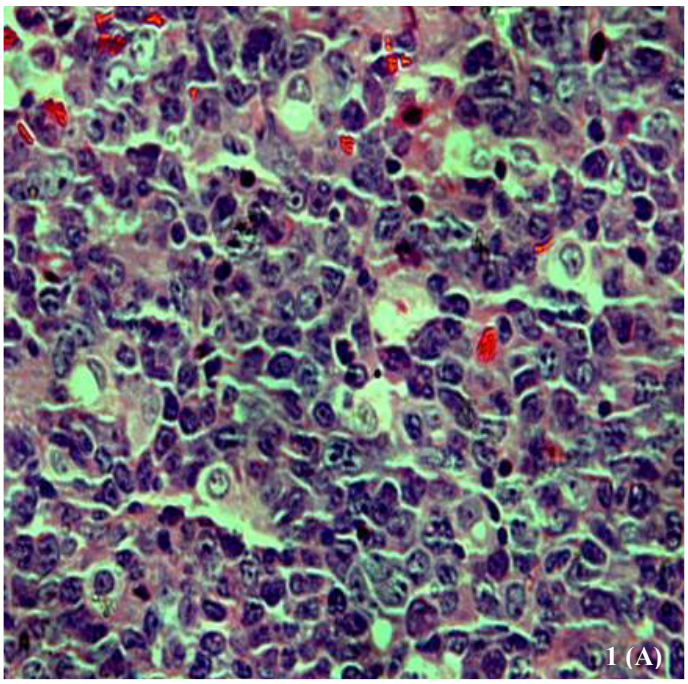


Figure 2 (A and B): The transesophageal echocardiography (TEE) shows the right atrial lymphomatous mass in figure 2 (A) and left atrial lymphoma with infiltration into the left ventricle in figure 2 (B).

The liver was just palpable 3 cm below the costal margin and the spleen was not enlarged. The ECG showed sinus rhythm with non specific ST-T changes.

The chest radiograph showed cardiomegaly, pulmonary congestion and bilateral mild pleural effusion. The hematological examinations showed normochromic normocytic anemia with mild thrombocytosis. Serum albumin and serum lactate dehydrogenase were 29.5g/L. and 641 U/L, respectively. Serologic screening test for human immunodeficiency virus was negative. Arterial blood gases showed respiratory alkalosis with mild hypoxia. 2-D echocardiography showed a left atrial mass infiltrating into the left ventricle with strikingly bright myocardium.



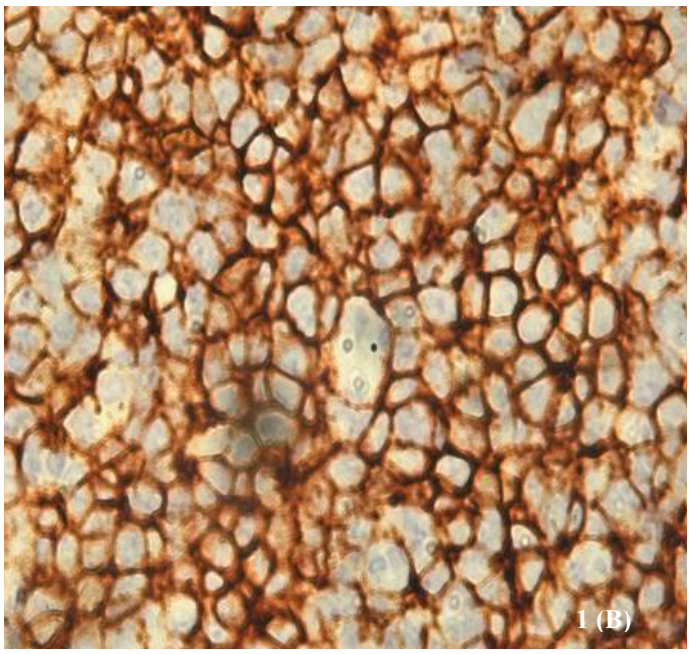


Figure- 3 (A and B): Tumor cells are composed of malignant, large, lymphoid cells arranged in diffuse manner as shown in figure 3A. The tumor cells are stained positive for CD20 in figure 3B.

A computed tomography (CT) scan of the chest demonstrated two filling defects in the right and left atria (Figure- 1A and 1B). Multiple lymphadenopathies were seen in the cervical, mediastinal, para-aortic and para-caval regions. The liver and spleen were of normal size.

Trans-esophageal echocardiography confirmed the location of the lymphoma mass in the bi-atrium with lymphomatous infiltration into the left ventricle (Figure 2A and 2B). The right atrial mass measured 3.9×2.9 cm and the left atrial mass measured 3.4×1.3 cm. The bone marrow and other organs did not show any evidence of tumour invasion. The histological examination revealed diffuse proliferation of lymphoid cells with high mitotic index and positivity for CD20. The histology confirmed a B-Cell non-Hodgkin's lymphoma of the heart. (Figure- 3 (A and B)

The patient received the CHOP regime chemotherapy (cyclophosphamide, doxorubicin, vincristine and prednisolone). He unfortunately succumbed to his illness on the 18th day of chemotherapy.

Discussion

Primary cardiac lymphoma is defined as a lymphoma exclusively involving the myocardium and/or pericardium. The majority of reported cases consist of B-Cell non-Hodgkin's lymphoma.(4, 8, 9) This tumour is an extremely rare malignancy which constitutes about 1.3% of primary cardiac tumour and 0.5% of extranodal lymphoma.(10) This condition is a disease exclusively found in the elderly men. The median age at presentation was 64 years old. Male to female ratio was 3:1.(5, 7) Patients with small secondary lesions in other parts of the body are also classified as primary cardiac lymphoma. Extensive extra cardiac involvement with the bulk of tumour within the pericardium or cardiac symptoms from lymphomatous cardiac infiltration at the time of diagnosis, have also been accepted as primary cardiac lymphoma.(4, 5, 7) Though some authors have strictly declared that the tumour is primarily localized in the heart structures, Carins et al, have reported that massive cardiac involvement with minimal infiltration into other sites is indicative of primary cardiac lymphoma.(11) Moreover, when cardiac lymphoma is bulky, it tends to metastasis. Our case merits to be considered as exclusively cardiac because the bulk of the tumour was mainly in the heart.

Cardiac lymphoma is usually located in the right atrium and less often in the left atrium or the left ventricle. In our present case, the tumour involved both the right and left atria with visible infiltration into the left ventricle. The tumour can also involve the inferior vena cava, superior vena cava, jugular vein and pulmonary artery. Intracardiac tumour can infiltrate all three layers of the heart.(4) In general, there are three possible mechanisms by which lymphoma can spread to other organs: direct infiltration, lymphatic or hematogenous spread.(2) It may seem difficult for primary cardiac lymphoma to directly extend into other structures due to cardiac movement. Up to now, only one case of direct extracardiac invasion has been reported.(12)

The involvement of sites like mediastinal and pleural most likely represent direct tumour spread.

Clinical presentation can vary from conduction defects to obstructive symptoms and death. The two most common clinical presentations are massive pericardial effusion and heart failure. Usual symptoms include heart failure unresponsive to diuretics, shortness of breath, palpitation, arrhythmias, cardiac tamponade, constitutional symptoms, syncopal attack and sudden death. The tumour may predispose to thromboembolic phenomenon. Other presentations include superior vena cava syndrome and acute myocardial infarction. Occasionally, aortic and tricuspid valvular involvement may cause hemodynamic decompensation and pulmonary hypertension.

The clinical progression is extremely rapid. Diagnosis is usually made late and most often made at postmortem examination. The prognosis is poor as antemortem diagnosis is extremely difficult. Appropriate diagnosis is of paramount importance prior to proper chemotherapy. Once the suspicion of cardiac mass has been made, a 2-D echocardiography is a good non-invasive diagnostic tool to detect the presence of tumour mass and pericardial effusion. Other investigative tools include computed tomography (CT) as well as cardiac magnetic resonance imaging (MRI). Cardiac MRI has been reported to have the diagnostic edge in the diagnosis of primary cardiac lymphoma. An isointense signal of the tumour to normal myocardium on T1- and T2- weighted images with heterogeneous signal enhancement have been described in primary cardiac lymphoma but have not been reported in other cardiac tumours.(5) The definitive diagnosis relies on tissue histopathological sample. Most reported cases are of diffuse B-Cell non-Hodgkin's lymphoma with majority large cell type. Occasionally, T cell lymphoma may be found. Cytogenetic staining of B cell lymphoma should be positive for CD20 as illustrated in this case.(4-5)

Treatment options in such patients include radiation and chemotherapy though the result is usually disappointing. This may be due to either late diagnosis or aggressive nature of the tumour. There is no evidence to indicate that surgery improves the patient survival. It is often difficult to completely resect the tumour. Combinations of Rituximab and conventional chemotherapeutic agents have shown promising result in some patients who have achieved complete remission.(4, 5, 13) The median survival would be only 1 month for patient without treatment. The median survival time of the patients who tolerate chemotherapy or radiotherapy is estimated to be one year. Rapid regression of intramural tumour may lead to rapid deterioration as a result of heart failure, arrhythmia or cardiac rupture. Early chemotherapy initiation resulted in meaningful clinical remission and delay disease progression at the same time. However, our patient unfortunately died on 18th day of chemotherapy. The exact cause of death is still unknown as his family refused to give their consent for the postmortem examination.

In conclusion, primary cardiac lymphoma is a rare entity and carries a poor prognosis. This tumour primarily affects elderly men and typically involves the right heart chambers. Prompt diagnosis and early initiation of treatment may result in clinically meaningful remission and prolong the survival time. The present case was unusual because both atria were involved which had rarely been reported in the literature.

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Central Nervous System Vasculitis in a Patient with Myelodysplastic Syndrome

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Abstract:
Myelodysplastic syndromes (MDS) are a group of hematological disorders ranging from chronic refractory anemia to leukemia. There are some reports about association of MDS with autoimmune disorders and vasculitis. In this study we describe a patient with MDS and vasculitis presenting with central nervous system (CNS) involvement. A clinical response to immunosuppressive therapy was also observed in the patient.

Key words: Anemia, Vasculitis, Cerebrovascular Accident

Introduction

Myelodysplastic syndromes (MDS) include a heterogeneous group of hematological disorders with quantitative changes of one or more blood and bone marrow elements.

There are some reports about association of MDS with autoimmune disorders such as polymyalgia rheumatica, relapsing polychondritis,(1) rheumatoid arthritis,(2) Sjogren syndrome,(1, 2) nephritic syndrome,(3) polymyositis,(4) Behcet disease(5) and some types of vasculitis such as cutaneous vasculitis,(1, 2, 4, 6-8) polyarthritis nodosa,(7, 8) Wegner granulomatosis, microscopic polyangitis, Henoch Schonlein purpura(8) and large vessel vasculitis.(9)

There is only one report of central nervous system vasculitis associated with myelodysplastic syndrome,(10) that small vessel vasculitis confirmed by necropsy in brain, lung, and kidneys.

We describe a patient with MDS and vasculitis presenting with CNS medium vessel involvement. A clinical response to immunosuppressive therapy was also observed in the patient.

Case report

An 18- year- old patient with a history of anemia was admitted to our hospital because of headache, dysarthria, and severe paresis of right upper limb. 5 years ago, she was considered as MDS (refractory anemia type) by bone marrow aspiration (myeloid/erythroid= 1/3). Her problems began 2 months before admission with headache and diplopia on left ward gaze. Sagittal sinus thrombosis and parietal lobe infarction were identified by MRI. Anticoagulation therapy was started 6 weeks later. In July, she experienced another cerebral attack with severe headache, dysarthria, convulsion and right upper limb paresis. The patient readmitted by neurologist and anticonvulsant therapy was added to her management. In this time she was referred to rheumatologist for more workup.

On clinical examination, the patient presented with severe dysarthria, diplopia, irritability and depressed mood and affect. The force of right upper extremity was as 0/5 but lower extremities were normal in strength. Vital signs included; blood pressure: 100/60 mmHg, pulse rate: 80 beat/min, temperature 36.5˚C and respiratory rate 16 breaths per minute. The remaining physical findings included ear, nose, throat, cardiovascular and pulmonary systems were all normal except for pale skin.

Routine laboratory tests included:

Hemoglobin (Hg): 7.3, white blood cell count (WBC): 2000, platelets count (Plt): 134000 and

erythrocyte sedimentation rate (ESR): 71. Serum creatinine: 0.4 and 24- hour collection of urine contained 170 milligrams protein. A bone marrow aspiration showed dyserythropoesis as nucleocytoplasmic dissociation, nuclei budding and binucleation and she was considered as probably MDS (figure 1). The Myeloid/Erythroid ratio was 0.5 (normal range: 1.5-4.5). To initially rule out an infectious disease, blood, urine, and bone marrow cultures did not show any pathogen. Serological tests for brucellosis and typhoid fever were negative. No antibodies to human deficiency virus or hepatitis B or C were detected. Transesophageal echocardiography did not show any vegetation and chest X ray, ultrasound of abdomen as well as pelvis did not yield any abnormality. The patient who underwent an MRI showed evidence of thrombosis, hemorrhagic and ischemic damage with edema. Lumbar puncture was not done because of mass lesion identified by MRI. In this time the patient was treated with dexamethasone, phenytoin, ceftriaxon, clindamycine and enoxaparine.

The results of immunology tests were negative for RF, Anti CCP, ANA, anti ds DNA, C and P ANCA, anti sm, anti Ro/SSA, anti La/SSB, anticardiolipin antibodies and direct and indirect coombs' test. Three days after admission, neurological deficits were progressed acutely with right facial and lower limb hemiparesis. Then subsequently, the patient's condition deteriorated. MRI findings according to disease course are shown in figure 2.

The treatment was started on IV pulse therapy with methyl prednisolone (1000 mg/day) for 3 days and then the patient was given cyclophosphamide (1000 mg) on 4th day. Two days after the beginning of IV methyl prednisolone, paresis in lower limb and face was improved and wonderfully force of right upper limb changed to 2/5. In this time, CBC included: Hg: 9.5, WBC: 4600, plt count: 206000 and erythrocyte sedimentation rate: 21. The treatment continued by oral prednisolone (1mg/kg/day) and monthly IV cyclophosphamid. In second month of treatment, the patient could speak fluently and force of upper limb was as 4-4.5/5. We tapered steroid but below 15 mg patient's headache and anemia became more severe, so this dose of prednisolone was continued. After 12 months cyclophosphamide was changed to azathioprine and after 3 months lab data included: Hg: 8.5, WBC: 5900, plt count: 191000 and erythrocyte sedimentation rate: 23.

Discussion

Vasculitis is a heterogeneous group of clinical disorders in which an inflammation of the blood vessel develops that may cause necrosis, aneurismal dilatation, stenosis, thrombosis and hemorrhage.(10) In practice, primary systemic vasculitides are classified according to their clinical presentations, histological features and the size of affected vessels.

CNS vasculitis is characterized by inflammatory damage to blood vessel walls in the brain and spinal cord and is classified as a vasculitis of medium-sized vessels,(11) resulting in symptoms and signs of CNS dysfunction. Primary angitis of the CNS (PACNS) is defined as inflammation of the cerebral vasculature without angiitis in other organs. The disease is more common in females and in case series the average age is about 42 years.(12, 13) The initial presentations are headache, encephalopathy and stroke. Moreover, multiple strokes may be found in varying ages.(11) In 2/3 of cases ESR is elevated.(14)

There are some reports about association of vasculitis and MDS,(1, 2, 4, 6-10) but there is only one case report of PACNS-like vasculitis.(10) Theories about mechanisms of autoimmunity and vasculitis associated with MDS include:

Immune deregulation and synthesis of auto antibodies due to abnormalities in T and B cells, immune complex- mediated due to impairment of macrophage- mediated clearance, defective neutrophil function and production of cytokines by abnormal lymphocytes and monocytes as well as prior exposure to chemotherapy or radiotherapy.(10)

MRI which is the most sensitive but not specific imaging method for detecting PACNS, is abnormal in approximately 90% of cases. The lesions appear as mass lesions or areas of signal change. It is reasonable to recommend brain biopsy for those who have a slow onset, severe neurologic impairment, striking cerebrospinal fluid abnormalities or for patients who have failed to respond to corticosteroid therapy.(11) The fulminated progression of neurologic deficits did not allow us to take brain biopsy but disease progression despite anticoagulation therapy, cytopenia, elevated ESR and dramatic response to immunosuppression therapy, are strong reasons for such an autoimmune phenomenon.

Combination therapy with prednisolone and cyclophosphamide for at least one year is recommended(1, 12, and 14) and early initiation of treatment improves the prognosis.(14)

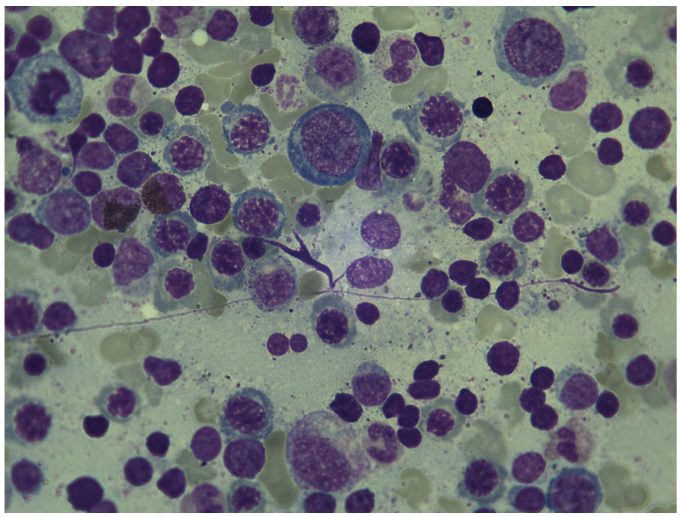

Figure- 1. Bone marrow aspiration showed dyserythropoesis as nucleocytoplasmic dissociation, nuclei budding and binucleation and she was considered as probably MDS.

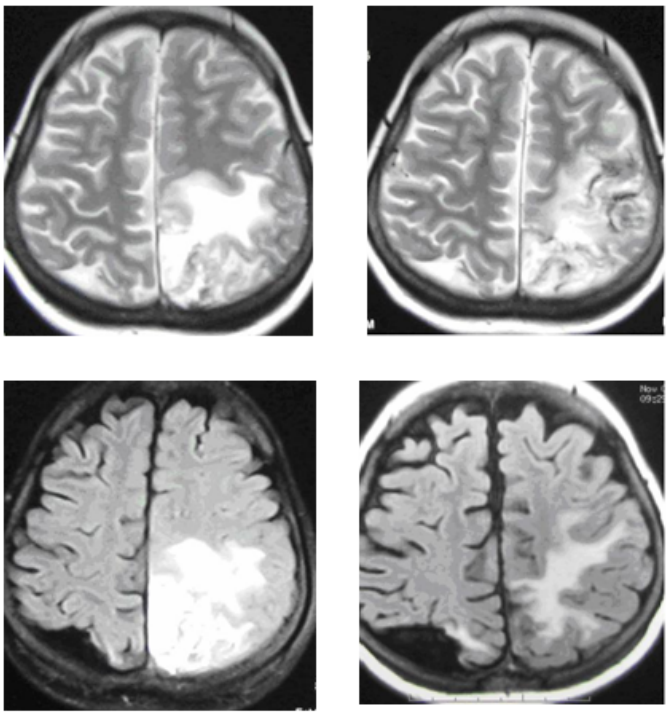

Figure- 2. MRI findings according to disease course are shown.

In this study we described a patient with MDS and PACNS-like vasculitis. According to our knowledge, it is the second report of CNS vasculitis in MDS in literature. We started prednisolone and cyclophosphamide early in the disease course. Due to young age, dramatic and persistent response to immunosuppression, the patient was different from other case reports.

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