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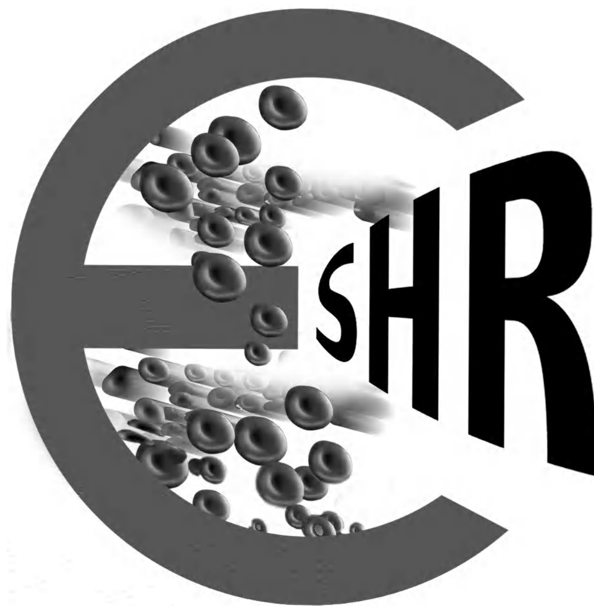

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CD133 Expression in Adult Egyptian Acute Leukemia Patients and its Impact on Disease Outcome

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ABSTRACT

Background: CD133 antigen is expressed restrictively by the immature subset of the CD34+ cells; hence it is expected to be a valuable prognostic marker in acute leukemia.

Aim: To assess CD133 expression frequency in patients with acute leukemia and to evaluate its relation to disease outcome.

Patients and Methods: The present study was carried on seventy-five newly diagnosed acute leukemia patients, recruited from hematology/oncology clinic of National Cancer Institute. The patients were divided into two groups, 24 acute lymphoblastic leukemia (ALL) and 51 acute myeloblastic leukemia (AML) patients. Patients were followed-up through out the period of the study (12 months). All patients were subjected to the complete history taking, thorough clinical examination and laboratory investigations, including complete blood count (CBC), Bone marrow (BM) aspirate examination, Immunophenotyping and assessment of CD133 expression using EPICS XL Flow Cytometer.

Results: CD133 was expressed in 28/75 patients (37.3%), 21/51 patients with AML (41.17%) and 7/24 patients with ALL (29.16%). The expression of CD133 was higher in AML than ALL without statistical significance (20.41±19.28 vs. 15.2±14.69; p=0.20). No significant correlation was found between CD133 +ve expression and the clinical data as regard sex, age, hepatomegaly, splenomegaly and lymphadenopathy in both ALL and AML. No significant correlation was found between CD133 +ve expression and the hematological data as regard, WBCs, Hb, platelets and peripheral blasts in both ALL and AML. There was significant positive correlation between CD133 and BM blasts in ALL (r=0.45; p=0.02), but insignificant in AML. In both ALL and AML, patients with CD133 +ve expression had statistically significant poor clinical outcome (relapse or death) (p=0.012 and 0.021). Patients with CD133+ve expression had shorter overall survival compared with CD133 –ve; this was significant in AML (8.95±0.69 vs. 10.3±0.54 months, p=0.05) and insignificant in ALL.

Conclusion: CD133-positive expression is a poor prognostic factor in adult acute leukemia and its expression could characterize a group of acute leukemic patients with resistance to standard chemotherapy, as well as high incidence of relapse and death. The use of CD133 as a prognostic marker in acute leukemia would be recommended to offer a chance for early intensive therapeutic intervention in cases designated as having poor prognosis.

Key Words: *AML – ALL – CD133.*

INTRODUCTION

Leukemia arises through the acquisition of genetic mutations in hematopoietic stem or progenitor cells, resulting in impairment of hematopoietic and unrestrained proliferation of an immature clone. The condition is lethal within a few months without treatment, but most young patients reach complete remission with chemotherapy. Many of them will relapse after a while, but an increasing number of young people survive for a long time [1]. Assessment of the prognosis of acute leukemia involves a number of clinical and laboratory criteria such as morphology, surface markers, cytogenetics and other recent criteria such as transcription factors and cytokines [2,3]. Prominin (CD133) is a membrane protein consisting of five membrane-spanning domains, two large N-glycosylated extracellular loops, an extracellular N-terminal domain and acytoplasmic C-terminal domain [4]. It has been initially isolated as a cell surface marker expressed on a subpopulation of CD34+ cells in hematopoietic stem and progenitor cells derived from human fetal liver, bone marrow and peripheral blood [5,6]. CD133 has been shown to be expressed in both acute and chronic myeloid leukemia and lymphoblastic leukemia in both adults and pediatrics [7]. Several studies

indicated that the CD133 antigen expression is related to CD34 cell surface expression may provide alternative, but similar information with regards to leukemic blast phenotype in acute myeloid leukemia (AML) [8,9]. However, in acute lymphoblastic leukemia (ALL), encouraging preliminary observations suggested that CD133 may provide an important marker capable of distinguishing normal stem progenitors from lymphoid leukemia initiating blasts [10,11]. The present study aimed to assess CD133 expression frequency in patients with acute leukemia and to evaluate its relation to disease outcome.

PATIENTS AND METHODS

Patients:

The present study included 75 newly diagnosed acute leukemia patients, recruited from hematology/oncology clinic of the National Cancer Institute, Cairo University. The study was approved by the Institutional Review Board (IRB) and a written informed consent was obtained from all cases before participation in the study. Patients were divided into two groups, 24 ALL (15 males and 9 females), their ages ranged from 18 to 65 with a mean of 37.12± 12.59 and a median of 34.5 years, and 51 AML (25 males and 26 females), their ages ranged from 25 to 83 with a mean of 40.87±16.3 and a median of 40 years. Patients were followed-up through out the period of study (12 months).

All cases were subjected to the following:

1- Complete history taking and thorough clinical examination.

2- Laboratory investigations:

Complete blood count (CBC) was done by the use of cell dyne-3700 (Abbott Diagnostics, Dallas, USA) with examination of peripheral blood (PB) stained smears for differential leucocytes count and blast cells percentage. BM aspiration and examination of stained smears was performed. Immunophenotyping and assessment of CD133 expression as well as CD133/ CD34 co expression in BM or PB samples were evaluated by the use of EPICS XL Coulter Flow Cytometer (Coulter-USA), (Fig. 1). Diagnosis of acute leukemia was based on the presence of blast cells ≥20% in BM film according to WHO proposal, together with presence of immunophenotyping results consistent with AML and ALL. Assessment of remission achievement was done after the induction of therapy by BM on day 14 and day 28 as well as follow-up of the patients through out the period of study with a range of 1 to 12 months and a median of 12 months.

Methods:

Fresh PB or BM samples were kept at ambient temperature and processed for immunophenotyping within 6 hours of collection. The EDTA anticoagulated BM and PB samples were diluted with phosphate buffered saline (PBS), pH 7.4 (Sigma Chemicals, St Louis), the final cell count suspension was adjusted at $10x10^3$/ml. For each sample, a set of tubes was prepared for a panel of fluorescein isothiocyanate (FITC)/phycoerythrin (PE) conjugated MoAbs used for diagnosis of acute leukemia including one for the isotypic matched negative control (supplied by Beckman Coulter, France), B-lineage markers (CD19, CD22, CD10), T-cell markers: (CD2, CD3, CD5, CD7), Myeloid markers: (CD13, CD33 and intracellular MPO), CD61, CD 41 and glycophorin in suspected cases. Monocytic marker: (CD14), and common progenitor's markers: (CD34, HLA-DR). PE labeled MoAb for detection of CD133 (Miltenyi Biotec, Germany).

Immunophenotypic analysis:

Immunophenotypic analysis was performed using whole blood staining method. Fresh samples were obtained from peripheral blood or bone marrow at the time of diagnosis. Double and Triple marker labeling was performed, including proper isotype controls. Samples were analyzed on Flow Cytometer (Coulter Epics, XL, Hialeh).

Detection of surface markers by direct staining:

The whole blood staining method was performed [12]. In short, 10µl labeled Mo Ab was added to 100µl whole blood (or BM), incubated in the dark for 20 minutes, hemolyzed by Lysing solutions, A11894, Beckman Coulter) and washed by PBS then analyzed.

Detection of intracellular markers:

Hundred µl of whole blood was lysed using lysis solution (Becton & Dickinson) for 10 minutes. Cells were washed once and re-suspended in 1ml PBS. A mixture of 500µl 4% paraformaldehyde as fixative, 500µl PBS and 5µl tween 20 as detergent was added to the cells

and incubated for 10min. The cells were washed and 10µl Mo Ab was added and incubated for 30min. at 4ºC. Cells were washed, re-suspended in 500 µl PBS and analyzed [12].

A minimum of 10000 events were acquired. Blast cell population was selected based on its forward and side scatter properties. The percentage of blast cells positive for the relevant studied marker was determined as a percentage from the blast cells population. The negative isotypic control was set at 0.5%. Cells were considered positive for a certain marker when ≥20% of cells expressed it, except for CD34 and intracellular MPO where its expression by 10% of cells was sufficient to confer positivity.

Statistical analysis:

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS-version 17). All data was expressed as mean±SD. For statistical evaluation, Student *t*-test was used. Significance was accepted at $p \leq 0.05$. Qualitative data were described in the form of number and percentage. Correlation Coefficient (*r*) was used for showing positive and negative correlation between variables. Quality of survival between studied groups was tested by Kaplan-Meier curve.

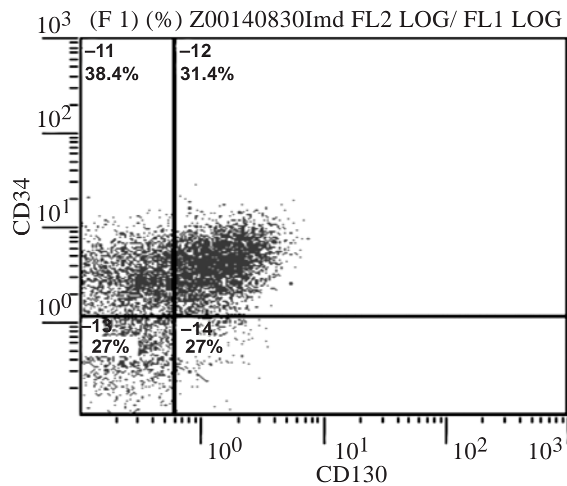

Fig. (1): Histogram showing co-expression of CD34 and CD133.

RESULTS

The present study was carried out on 75 newly diagnosed acute leukemia, 24 ALL and 51 AML. The demographic, clinical and hematological data of the studied ALL and AML patients are presented in (Tables 1,2).

Table (1): Demographic and clinical data of 75 acute leukemia patients.

Parameter	ALL (No.=24)	AML (No.=51)
Age:		
Mean±SD	37.12±12.59	40.87±16.3
Range	18-62	18-83
Median	34.5	40
Gender: No (%):		
Male	15 (62.5)	25 (49)
Female	9 (37.5)	26 (51)
Hepatomegaly:		
No. (%)	8 (33.3)	231 (60.78)
Splenomegaly:		
No. (%)	8 (33.3)	29 (56.86)
Lymphadenopathy:		
No. (%)	17 (70.8)	21 (41.18)

ALL : Acute lymphoblastic leukemia.
AML : Acute myeloid leukemia.

Table (2): Hematological data of 75 acute leukemia patients.

Parameter	ALL (No.=24)	AML (No.=51)	*p*
TLC: x10^9/L	46.2±45.57 (1.13-273)*	39.5±38.59 (1.1-252)	0.5
Hb: g/dl	7.6±1.47 (5.3-12)	7.8±3.22 (2.6-25)	0.8
Platelets: x10^{12}/L	67.16±57.6 (5-256)	52.68±52.29 (2-260)	0.2
PB blasts	85±13.1 (44-97)	71.0±15.2 (33-97)	<0.001
BM blasts	78±20.1 (5-98)	79.0±12.9 (45-98)	0.824

ALL : Acute lymphoblastic leukemia.
AML : Acute myeloid leukemia.
* Mean±SD (range).

CD34 was expressed in acute leukemia with higher expression in ALL than AML (42.97%±32.09 vs 27.63%±26.71, p=0.046). CD133 was positive in 28/75 (37.3%) patients with acute leukemia, 21/51 (41.17%) with AML and 7/24 (29.16%) with ALL, its expression was insignificantly higher in AML than ALL (20.41±19.28 vs 15.2±14.69; p=0.20). CD133 expression was restricted only to CD34 positive cells; all CD133 positive cells expressed CD34 (Fig. 1).

There was positive correlation between CD34 and CD133, significant in ALL (Fig. 2) and insignificant in AML patients (r=0.55, p= 0.001 and r=0.15, p=0.27 respectively).

Both groups showed no significant correlation between CD133 on one side and clinical data as regards sex, age, hepatomegaly, splenomegaly

or lymphadenopathy on the other side (Table 4). As regards hematological parameters significant positive correlation was encountered between CD133 and BM blasts in ALL and insignificant in AML (r=0.45; p=0.02 and r=0.04, p=0.78 respectively), other parameters showed insignificant correlations in both groups.

As regards the clinical outcome the percentage of remission was significantly higher among patients with CD133 negative than patients with CD133 positive expression in both groups (p= 0.012, 0.021). On the other side, the percentage of relapse and death was significantly higher in patients with CD133 positive than patients with CD133 negative in both groups (p=0.012, 0.021, Table 3).

Table (3): Impact of CD133 expression on the outcome of 75 acute leukemia patients.

Parameter	No.	Remission		Relapse		p value
		No.	%	No.	%	
ALL	24	17	70.8	7	29.2	
CD133 –ve	17	14	82.35	3	17.65	0.012
CD133 +ve	7	3	42.9	4	57.1	
AML	51	30	58.8	21	41.2	
CD133 –ve	30	22	73.33	8	26.67	0.021
CD133 +ve	21	8	38.1	13	61.9	

Patients with positive CD133 expression had shorter overall survival (OS) compared to CD133-negative patients; the difference was significant in AML (8.95±0.69 vs. 10.3±0.54 months, p=0.05, Fig. 3) and insignificant in ALL (9.16±0.77 vs. 10.6±1.67 months, p= 0.3, Fig. 4).

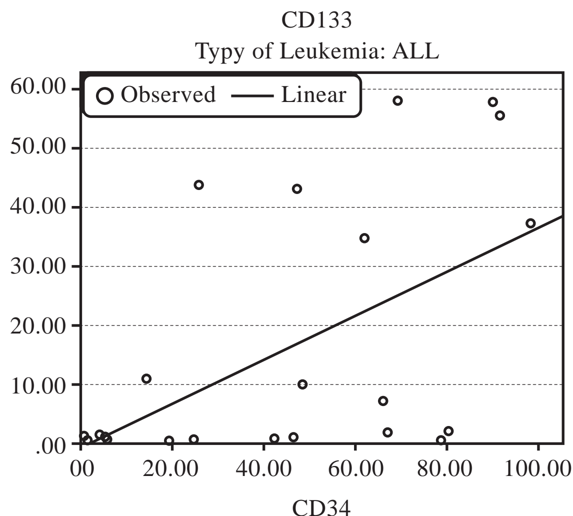

Fig. (2): Correlation between CD133 and CD34 in ALL.

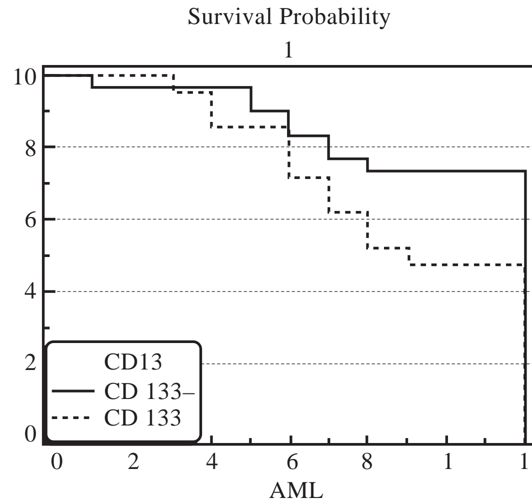

Fig. (3): Kaplan-Meier survival curve comparing survival in CD133 negative cases and CD133 positive in AML patients.

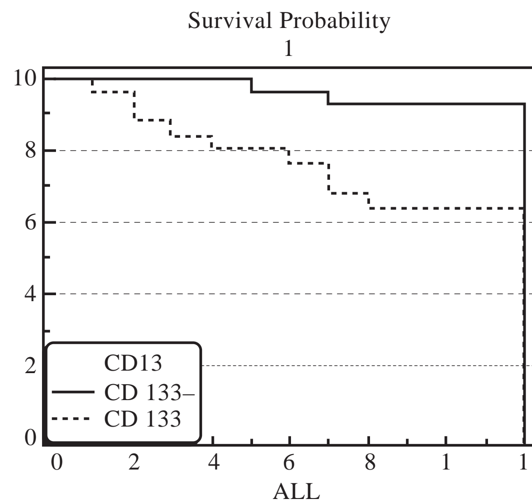

Fig. (4): Kaplan-Meier survival curve comparing survival in CD133 negative cases and CD133 positive in ALL patients.

DISCUSSION

Leukemia arises through the acquisition of genetic mutations in hematopoietic stem or progenitor cells, resulting in impairment of normal hematopoiesis and unrestrained proliferation of an immature clone. The condition is lethal within a few months without treatment, but most young patients reach complete remission with chemotherapy. Many of them will relapse after a while, but an increasing number of young people survive for a long time [1].

Given the fact that CD133 antigen is expressed restrictively in the more immature subset of the CD34 cell population, we could expect

the CD133 antigen to be more valuable prognostic marker compared to the CD34 antigen. It has also been suggested that, like the CD34 antigen, expression of the CD133 antigen in acute leukemia could be correlated with either a more immature phenotype of the blast population or to a bad prognosis [13].

In this study CD34 showed higher expression in ALL than AML with statistical significance; this result is in agreement with Filler [9], who reported that CD34 antigen is expressed in a relatively high proportion of cases ranging from 30 to 60% in AML and from 60 to 70% in ALL, and that its presence has been related to more immature morphological and immunophenotypic features, as well as a poorer prognosis.

In this study, CD133 was expressed in 37.3% of acute leukemia; 41.17% in AML and 29.16% in ALL groups. These results are in agreement with previous reports [8,14-17]. However Cox and coworkers [7] stated that there have been conflicting reports on the expression of AC133 in ALL, whereas some found high level of CD133 in particular cases [8-11], others detected only low levels [5] or none at all [13]. The frequency of CD34 expression was proved to be found on 100% of CD133-positive cases, this result confirmed that CD133 expression is restricted to CD34 positive cells and this is in agreement with previous reports [14,19]. We detected positive correlation between CD133 and CD34 expression significant in ALL and insignificant in AML; this is in agreement with previous reports [18,19].

To elucidate the value of CD133 expression as a prognostic factor in acute leukemia, we investigated the significance of its expression in relation to various clinical, laboratory and standard prognostic factors, as well as to clinical outcome of patients. No significant correlation was noted between age of patients and positivity of CD133 expression in acute leukemia patients, this is in agreement with previous reports [13-17]. In contrast to our study Wuchter and coworkers [8] demonstrated that there was a weak inverse correlation between CD133 positive expression and age of ALL patients.

In our study we found that no significant association was detected between the gender and the CD133 positive expression, which is in agreement with previous results [13-16]. No association was detected between clinical variables (hepatomegaly, splenomegaly and lymphadenopathy) and CD133 expression; this is in agreement with other reports [10,14-16].

Several studies reported that there were insignificant correlation between CD133 positive expression and hematological data of patients with acute leukemia [10,14,16,18-20], those results are in agreement with our result as regards hemoglobin concentration, WBCs, platelet counts and percentage of leukemic blast cells in PB, but in contrast we found that there was significant positive correlation with BM blasts in ALL. Elgendi et al., [15]. Reported that there was significant positive correlation between CD133 positive expression and higher percentage of BM and peripheral blasts and this is in partial agreement with our results.

As regards outcome, we found that the percentage of remission among patients with CD133 negative was significantly higher than patients with CD133 positive expression in both ALL and AML. The other way round, the percentage of relapse and death in patients with CD133 positive was significantly higher than patients with CD133 negative in both groups. These results are consistent with previous reports who found a trend towards higher complete remission (CR) rates in CD133-negative acute leukemia cases when compared to CD133-positive ones [15,16,20] and a tendency for poorer outcomes in CD133-positive acute leukemia compared to CD133-negative ones [13,15].

In the current study, the unfavorable prognosis conferred by CD133 expression was also reflected on the OS for patients. This was previously reported by some authors who proved the association between CD133 expression and shorter remissions in acute leukemia patients [13,15,16,20] but denied by others who reported that the increased expression of this protein was not significantly associated with a shorter OST [10,14,23]. This discrepancy between the studies could be attributed, at least partially, to the difference in the methods of detection of CD133 and the therapeutic protocols applied to the patients.

In conclusion, CD133 expression was highly associated with poor prognosis in acute leukemia patients. It may be considered an adverse prognostic factor; its expression could characterize

a subgroup of acute leukemia patients with higher resistance to standard chemotherapy, relapse or death. Accordingly, it is recommended to add CD133 to acute leukemia workup panel to offer a chance for early intensive therapeutic intervention in cases designated as having poor prognosis.

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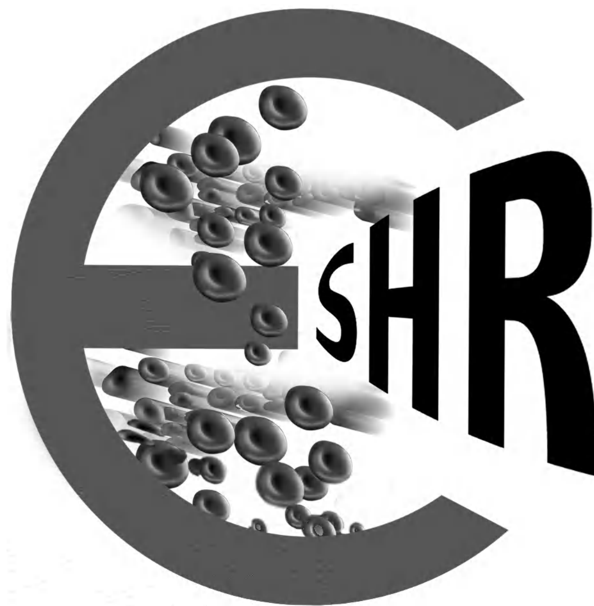

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Impact of Multidrug Resistance Gene 1 (MDR1) C3435T Polymorphism on Chronic Myeloid Leukemia Response to Tyrosine Kinase Inhibitors

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ABSTRACT

Background: Single nucleotide polymorphisms (SNPs) of multiple drug resistance (MDR1) gene are associated with altered P-glycoprotein (p-gp) activity and contribute to resistance to tyrosine kinase inhibitors (TKIs) in chronic myeloid leukemia (CML).

Objectives: We aimed to demonstrate the association between MDR1 gene C3435T polymorphism and molecular response in newly diagnosed chronic phase (CP) CML patients to standard dose upfront imatinib and nilotinib therapy.

Patients and Methods: MDR1 C3435T was genotyped using polymerase chain reaction Restriction Fragment Length Polymorphisms (PCR-RFLP) at diagnosis. BCR-ABL1 transcripts level was measured by Real Time Quantitative polymerase chain reaction (RQ-PCR) at diagnosis then every 3 months.

Results: This study included 74 Philadelphia (Ph') positive CP-CML patients; 38 males and 36 females. Median age at diagnosis was 38 years (18-78). Median BCR-ABL1 level was 101%. Forty patients received imatinib (54%) while 34 received nilotinib (46%). Optimal response at 12 month was 35% in the imatinib arm versus 80% in the nilotinib arm (p=0.001). The frequency of MDR1 SNP C3435T was 46%, 32% and 22% for CC, TT and CT genotypes, respectively. Optimal response at month 12 differed significantly between imatinib and nilotinib among patients with MDR1 3435TT genotype (11% versus 83%, respectively, p=0.002) while less significant difference was found between the two drugs in CC and CT genotypes (35% vs. 75% and 60% vs. 83%, respectively, p=0.042 & p=0.588).

Conclusion: MDR1 3435TT may be used as an additional criterion for initiating nilotinib instead of imatinib as front line therapy for CP-CML patients. We demonstrated the usefulness of MDR1 SNP polymorphism in the identification of CML patients who may or may not respond optimally to imatinib.

Key Words: *CML – SNP – MDR1 gene – C3435T – Molecular response.*

INTRODUCTION

Despite of striking efficacy of tyrosine kinase inhibitors (TKIs) in treatment of chronic phase chronic myeloid leukemia (CP-CML), resistance develops over time in many patients **[1]**. One of the limitations in CML treatment is the development of multidrug resistance (MDR1), a well-known mechanism responsible for drug resistance by over expression of ABCB1 transporter genes **[2]**. This gene encodes P-glycoprotein (P-gp) that transports anti-leukemia drugs out of the cell. MDR1 product is an ATP-driven efflux pump contributing to the pharmacokinetics of drugs that are P-gp substrates. Imatinib and nilotinib have been reported as substrates of P-gp-mediated efflux **[3-5]**.

MDR1 gene polymorphisms alter the expression level of P-gp and consequently result in drug resistance. Variations in the MDR1 gene product can directly affect the therapeutic effectiveness **[6]**. The up-regulation of drug transporter (ABCB1) is one of the specific causes of resistance to TKIs. SNPs in ABCB1 gene have the potential to alter protein function and also influence the efficiency of absorption or elimination of BCR-ABL inhibitors **[7]**.

More than 50 single nucleotide polymorphisms (SNPs) have been identified, so far, in MDR1 gene by use of polymerase chain reaction Restriction Fragment Length Polymorphism

(PCR-RFLP). These SNPs affect the expression and function of the P-gp in many ways. MDR1 C3435T is associated with altered P-gp activity and is considered one of the most important MDR1 gene polymorphisms as it was demonstrated to be the main functional polymorphism affecting mRNA stability [8-10]. The polymorphism at exon 26 C > T at 3435 position is a synonymous polymorphism which contributes to the change in substrate selectivity of P-gp, without any significant change in protein expression levels [9].

Our main objective was to investigate the possible influence and association between MDR1 gene polymorphism C3435T and molecular response to newly diagnosed CP-CML Egyptian patients receiving upfront standard doses of either imatinib or nilotinib.

PATIENTS AND METHODS

Study population: The study included 74 CP-CML patients referred to the Clinical Hematology Unit, Kasr Al-Ainy Hospital, Cairo University in the period February 2012 to March 2013. Patients were followed for 12 months to evaluate their response to TKI therapy and its association with MDR1 C3435T polymorphism. The study was carried out according to the declaration of Helsinki and approved by the Institution Review Board. All patients provided written informed consents.

CP CML was defined according to WHO criteria [11]. Exclusion criteria were: Age <18 years, pregnant females, Ph' negative CML and patients previously treated with interferon-α. All patients were submitted at diagnosis to detailed medical history recording, complete physical examination, bone marrow aspirate (BMA), karyotype, complete blood count (CBC) with examination of peripheral blood film, estimation of Sokal and Hasford risk scores in addition to quantitative measurement of BCR-ABL1 transcripts by Real Time Quantitative polymerase chain reaction (RQ-PCR) at diagnosis and every 3 months interval. MDR1 gene C3435T was genotyped using PCR restriction fragment Length polymorphisms (PCR-RFLP). Major molecular response (MMR) was defined as a 3 log reduction of BCR-ABL1 transcripts level, corresponding to ≤0.1% on international scale (IS). Response to treatment was evaluated according to the 2013 European Leukemia Net (ELN) criteria [12]. All patients received either imatinib 400mg od or Nilotinib 300mg bid, orally.

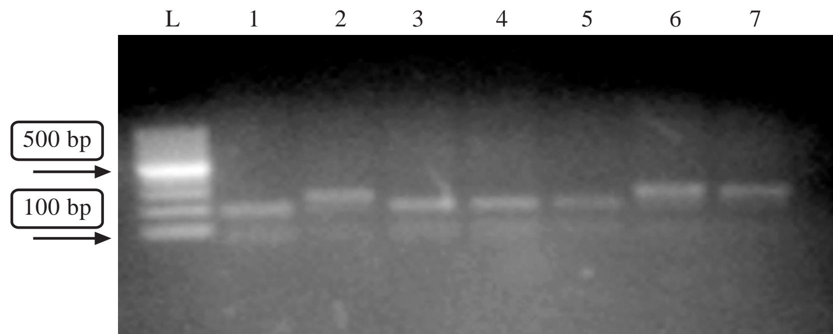
BCR-ABL1 transcripts measurement: Total RNA extraction was carried out from 5ml fresh peripheral blood leucocytes according to the initial silica extraction method using QlAmp RNA blood minikit (Qiagen, Hielden, Germany). For cDNA synthesis, 1µg total RNA from each sample was used to synthesize first-strand cDNA with random primers in a 20ul mix using MMLV reverse transcription enzyme (40U/ul) and RNasi Stop RNAses inhibitor (4U/ul) (RT-kit Plus, Nanogen). RT cycles consisted of 25ºC at 10min (primer annealing), 37ºC at 45min (reverse transcription step) and 93ºC at 3min (final denaturation).

BCR-ABL1 transcripts level was determined using RQ-PCR detection system (Ecco Illumina, USA). The ABL1 control gene and the BCR-ABL1 target gene were measured using Universal PCR master mix (Real Time alert QPCR, Nanogen) and specific primers and hydrolysis probes (Philadelphia p210 and Abl QPCR Amplimix and Ampliprobes). PCR mix (25ul) for ABL1 housekeeping gene and BCR-ABL1 target gene consisted of: 12.5ul Amplimaster (Tris HCl, MgCl2, dNTPs, Rox, Uracil–N–glycosidase, Taq DNA polymerase), 1.25ul amplimix (oligonucleotide primers), 1.25ul Ampliprobe (oligonucleotide fluorescent probe), 5ul distilled water and 5ul cDNA samples and standards. Quantitations were made against a set of 4 plasmid based p210 and ABL1 standards (Philadelphia p210 QPCR standards, Nanogen) of known concentrations (10^5, 10^4, 10^3 and 10^2 copies) used as positive control in each run as well as to generate a standard curve for the amplification assay. Cyclic conditions consisted of UNG decontamination 50ºC for 2min, initial denaturation 95ºC for 10min and 45 cycles of amplification at 95ºC for 15sec and annealing/ extension at 60ºC for 1min. A set of reference RNA (4 samples) (Nanogen) with high and low BCR-ABL1 and ABL1 levels were processed in the same way as patient's samples and was used to convert results into International Scale (IS). Calculated conversion factor was 1.1 in our laboratory. Any result with ABL Ct >29 or undetermined was considered invalid.

MDR1 C3435T genotyping: Genomic DNA was extracted from peripheral blood according

to manufacturer instructions by salting out method using Gentra Puregene Blood kit (Qiagen, Hielden, Germany). The sequence of primers used for MDR1 exon 26 detection were: 5'-GCTGGTCCTGAAGTTGATCTGTGAAC-3' as forward and 5'ACATTAGGCAGTGAC-TCGATG AAGGCA-3' as reverse primer. The PCR reaction was performed in a final volume of 50ul including 1X buffer with 1.5mM MgCI2, 1µM of each primer, 200µM of each dNTP (Sigma), 2.5 units Hot start Taq DNA polymerase (5u/µL, Fermentas) and 200ng of genomic DNA. PCR amplification consisted of initial denaturation at 94ºC for 5min followed by 35 cycles of denaturation at 94ºC for 30sec, annealing at 61ºC for 30sec, and extension at 72ºC for 30sec. Final extension was performed at 72ºC for 4min. Amplified segments were analyzed by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and observed under ultraviolet light.

The PCR product (248 bp in size) was digested for 3h at 37ºC with 2U MboI restriction enzyme (Bioron). DNA fragments generated after restriction enzyme digestion were analyzed by electrophoresis on 2% agarose gel, stained with ethidium bromide and observed with an ultraviolet transilluminator (Fig. 1). The expected fragments sizes were: 238-bp fragment for TT genotype (T allele abolished restriction site), 172- and 60-bp fragments for the CC genotype, and 238-, 170- and 60-bp for the CT genotype [6,13].



L: 100 bp ladder. Lanes 1, 3, 4, 5: CC genotype. Lane 2, 6, 7: CT genotype

Fig. (1): Electrophoresis of Mbol digested PCR product generated by amplified genomic DNA using MDR1 specific primers.

Statistical analysis:

All analyses were performed using the statistical package for social sciences (SPSS software version 21. Comparison between groups was performed using independent sample *t*-test or one way ANOVA for quantitative variables. Chi square and Fisher's exact tests were used for qualitative data. *p*-values <0.05 were considered statistically significant [14].

RESULTS

Patients: This study included 74 Ph' positive CP-CML patients; 38 males (51%) and 36 female (49%). Median age at diagnosis was 38 years (18-78). According to Sokal risk scoring; 29/74 (39%) were high risk, 25/74 (34%) were intermediate and 20/74 (28%) were low risk group. On applying Hasford scoring system; 20/74 (37%) were high risk, 26/74 (35%) were intermediate and 20/74 (28%) were low risk. Both scores were correlated (p=0.001).

Treatment: 40 (54%) patient received imatinib while 34 (46%) received Nilotinib. Four patients on nilotinib were lost follow-up. One patient (1/43, 2%) stopped imatinib after 3 month due to grade 4 hematological toxicity and was shifted to 2nd generation TKls. Three patients (3/30, 10%) stopped nilotinib after 6 months (1 due to persistent grade 2 hepatotoxicity; 2 due to grade 4 hematological toxicity). Two patients treated with imatinib (4.5%) progressed to blastic crisis 3 months after starting treatment while no patient progressed in the nilotinib arm.

BCR-ABL1: BCR-ABL1 transcripts level at diagnosis was 150.8%, ±140 with a range of 32-605 and a median of 101%; range between

25th percentile & 75th percentile was 55.5-208%. A significant higher optimal response rate was achieved in the nilotinib arm at month 12. Median BCR-ABL1 transcripts level was 0.1% at month 12 in the nilotinib arm versus 2% in the imatinib arm. Patient's response to both TKIs is shown in (Table 1). Kinetics of molecular responses is shown in (Table 2).

Table (1): Response of CP-CML patients to treatment with TKIs.

Parameter	Imatinib		Nilotinib		p value
	N	%	N	%	
Response at 3 months:					
Optimal	14/40	35	17/30	56	
Warning	24/40	60	12/30	41	0.196
Failure	2/40	5	1/30	3	
Response at 6 months:					
Optimal	14/37	38	18/30	60	
Warning	7/37	19	6/30	20	0.109
Failure	16/37	43	6/30	20	
Response at 12 months:					
Optimal	13/37	35	24/30	80	
Warning	5/37	13	4/30	13	<0.001
Failure	19/37	52	2/30	7	

CP-CML: Chronic phase-chronic myeloid leukemia.

Table (2): Kinetics of molecular response of CP-CML patients to treatment with TKIs.

Time of testing	Drug	No.	Median (%)	Range	p value
BCR-ABL1% M3	Imatinib	40	21.5	0.0-560.0	0.040
	Nilotinib	30	8.0	0.0-382.0	
BCR-ABL1% M6	Imatinib	37	6.7	0.0-287.0	0.070
	Nilotinib	30	1.0	0.0-225.0	
BCR-ABL1% M9	Imatinib	37	3.0	0.0-110.0	0.006
	Nilotinib	30	0.3	0.0-55.0	
BCR-ABL1% M12	Imatinib	37	2.0	0.0-157.0	0.001
	Nilotinib	30	0.1	0.0-18.0	

CP-CML: Chronic phase-chronic myeloid leukemia.
M: Month.

MDR1 C3435T genotypes: 74 CML patients were genotyped for MDR1 C3435T allele in addition to 72 normal controls. MDR1 CC genotype was the most frequent among patients and normal controls (Table 3).

Optimal response at month 12 was significantly higher in nilotinib arm among patients carrying the TT genotype, however, the difference was less but still significant in CC genotype and no difference was found between both TKIs in the CT genotype group. Difference in response achieved between the 2 drugs in relation to individual MDR1 C3435Tgenotypes is shown in (Table 4).

Table (3): MDR1 C3435T genotype and allele frequency distribution in CML patients and controls.

	Patients (n=74)	Controls (n=72)	p value
C3435T genotype (n, %):			
CC	34 (46%)	44 (61%)	
CT	16 (22%)	12 (17%)	
TT	24 (32%)	16 (22%)	0.180
Allelic frequencies (%):			
C allele	57%	68%	
T allele	43%	32%	0.230

CC : Homozygous 3435C genotype.
CT : Heterozygous C 3435T genotype.
TT : Homozygous 3435T genotype.
C : 3435C allele.
T : 3435 T allele

Table (4): Impact of C3435T genotypes on the response of CP-CML patients to imatinib and nilotinib.

Time point	C3435T genotypes						p1-value	p2-value	p3-value
	Imatinib			Nilotinib					
	TT No. (%)	CT No. (%)	CC No. (%)	TT No. (%)	CT No. (%)	CC No. (%)			
Month 3:									
Optimal	2 (17%)	5 (62%)	7 (35%)	6 (60%)	5 (63%)	6 (50%)			
Warning	9 (75%)	3 (37%)	12 (60%)	4 (40%)	3 (37%)	5 (42%)	0.074	1.00	0.473
Failure	1 (8%)	0 (0%)	1 (5%)	0 (0%)	0 (0%)	1 (8%)			
Month 6:									
Optimal	1 (10%)	5 (62%)	8 (42%)	5 (50%)	5 (71%)	8 (61%)			
Warning	4 (40%)	0 (0%)	3 (16%)	3 (30%)	1 (14%)	2 (15%)	0.129	1.00	0.497
Failure	5 (50%)	3 (37%)	8 (42%)	2 (20%)	1 (14%)	3 (23%)			
Month 12:									
Optimal	1 (11%)	6 (60%)	6 (35%)	10 (83%)	5 (83%)	9 (75%)			
Warning	2 (33%)	1 (10%)	2 (12%)	1 (8%)	1 (17%)	2 (17%)	0.002	0.59	0.042
Failure	6 (55%)	3 (30%)	9 (53%)	1 (8%)	0 (0%)	1 (8%)			

CP-CML: Chronic phase-chronic myeloid leukemia. p1-value for TT. p2-value for CT. p3-value for CC.

DISCUSSION

Resistance to BCR-ABL1 inhibitors is a pressing challenge in the treatment of CML. Although BCR-ABL1 point mutations and additional cytogenetic abnormalities are the main mechanisms of resistance, MDR1has been acknowledged among the mechanisms of resistance. The action of MDR1 is to reduce intracellular drug accumulation through Pgp-mediated efflux thus hampering the achievement of effective drug levels at the target site [7]. The possible importance of MDR1 SNPs has been recently appreciated.

The ENESTnd study, testing nilotinib 300mg twice daily versus imatinib 400mg once daily, reported a significantly higher rate of MMR after 1 year (50% vs. 27%) and 3 years (73% vs. 53%), and a significantly higher rate of MR 4.5 after 3 years (32% vs. 15%) in favor of nilotinib [15]. In this study, patient's response was assessed by RQ-PCR measurements of BCR-ABL1 mRNA transcripts level on IS. Early achievement of MMR on nilotinib included significantly higher rates of optimal responses at all-time points. According to ELN 2013, MMR rates at month 12 in our patients were higher in nilotinib than imatinib arm (80% vs 35%, $p<0.001$). Nilotinib was superior to imatinib in reduction of BCR-ABL1 transcripts level at month 3 (8% vs 21.5%, $p=0.040$), month 6 (1% vs 6.7% with $p=0.070$), month 9 (0.3% vs 3%, $p=0.006$) and month 12 (0.1% vs 2%, $p=0.001$). No patients on nilotinib progressed to accelerated phase or blastic crisis which is consistent with previous data that linked achievement of response to maintaining response and protection from progression [16].

In this study, we observed a higher frequency of MDR1 3435TT genotype in CML patients than healthy controls. When the distribution of genotype frequency was analyzed as regards MMR at month 12, TT genotype patients had a significantly higher optimal response rate in nilotinib arm when compared to imatinib (83% vs 11%, $p=0.002$). Difference in response between both TKIs was less but still significant in CT ($p=0.042$) while no difference was observed in CC genotype ($p=0.588$). Patients with the least optimal response to imatinib at month 12 were those carrying the TT genotype. Interestingly, 2 of these patients transformed into acute leukemia. The lower response to imatinib therapy in our 3435 TT patients was almost similar to others who correlated higher frequency of TT genotype with failure to achieve hematological response [17].

Results of this study differ from reports that showed decreased level of P-gp mRNA expression and the 3435 T allele [18] in addition to the lower activity of the T allele of the silent 3435C>T polymorphism [9]. Another study showed no difference regarding MMR rates among patients with CC, CT and TT genotypes [19]. However, in our patients' cohort, resistance to imatinib was proven to associate with the number of T alleles being more evident in the homozygous TT genotype. In consistence with other data [10], our results show that there was a significantly higher resistance to imatinib in 3435TT carriers whereas CC and CT genotypes exhibit different prognosis. This may suggest that CML resistance may arise, among other causes, from an association with homozygous T allele.

Before starting TKI therapy, MDR1 gene polymorphism pattern testing in individual CML patients may be important in determining treatment strategy. Studies in CML patients showed different results regarding MDR1 SNP and it is obvious that racial differences can affect the frequency and effect on treatment of these polymorphisms [18].

In conclusion, we demonstrated a significant correlation between MDR1 3435TT genotype and lower imatinib response in our CP-CML patients. We suggest that this may be used as an additional criterion for initiating nilotinib instead of imatinib as front line therapy for these patients. We need to expand this work on a larger scale population to prove the resistant effect of MDR1 3435TT genotype on imatinib and its therapeutic relevance for newly diagnosed CP-CML Egyptian patients.

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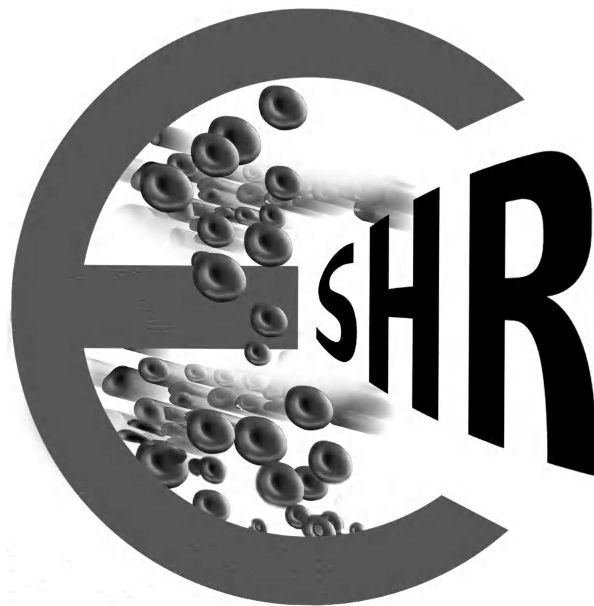

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The Efficacy of Procalcitonin in Early Detection of Sepsis in Patients Undergoing Bone Marrow Transplantation

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ABSTRACT

Background: Infections are among the foremost causes of non-relapse mortality in hematopoietic cell grafts recipients and can cause significant morbidity, both in the early and late transplant periods.

Objective: To evaluate the efficacy of serum procalcitonin (PCT) level in early detection of sepsis in patients subjected to hematopoietic stem cell transplantation (SCT).

Patients and Methods: Thirty subjects, from Department of Hematology and Stem Cell transplantation, Maadi Armed Forces Medical Compound, divided into three groups were enrolled. Group I: 10 adult patients with allogenic bone marrow transplantation (BMT). Group II: 10 adult patients with autologous BMT. Group III: 10 healthy adult volunteers, age and sex matched, as controls. Serum PCT was detected by chemiluminescence immunoassay in day 5, day 10 and day 15. Blood cultures were done to all patients during the period of transplantation when sepsis was suspected.

Results: In all the 20 (100%) patients, serum procalcitonin level was positive. In patients with autologous SCT, there were 5 patients (50%) with negative and 5 (50%) with positive cultures. In allogenic stem cell transplantation, there were 2 patients (20%) with negative and 8 (80%) with positive cultures.

Conclusion: PCT is more sensitive and rapid to detect sepsis in the patients as the procalcitonin could detect sepsis in the cases with negative cultures and it was more correlated to fever than cultures in these patients.

Key Words: *Procalcitonin (PCT) – Stem cell transplantation (SCT) – Sepsis – Culture.*

INTRODUCTION

Stem cell transplantation has been described as both intensive investigational therapy for end-stage disease and as standard curative treatment for malignant and non-malignant conditions [1].

Infections are among the foremost causes of non relapse mortality in HSCT recipients and can cause significant morbidity, both in the early and late transplant periods. Immune defects occurring in the post transplant period can be divided into predictable phases based on time from engraftment [2]. Procalcitonin (PCT) is a 116 amino acid prohormone of the hormone calcitonin. PCT can be produced by several cell types from a wide range of organs in response to inflammation or infection [3-5].

In patients, after near complete eradication of the leukocytes population by chemotherapy, a microbial infection still induces a ubiquitous increase of CALC-I gene group expression leading to the release of PCT from tissues. Consequently, PCT levels still increase several thousand-fold in severe systemic infections in immuno-compromised patients [6-7]. PCT levels have prognostic implications as persistently elevated or increasing levels can be associated with adverse outcome [8-9].

The aim of our study was to evaluate the diagnostic accuracy of PCT in early detection of sepsis in patients undergoing HSCT.

PATIENTS AND METHODS

Patients: The study was carried out on 30 subjects from the Department of Hematology and Stem Cell transplantation, Maadi Armed Forces Medical Compound.

Group I: 10 adult patients were subjected to allogeneic SCT. Group II: 10 adult patients

were subjected to autologous SCT. Group III: 10 healthy adult volunteers, age and sex matched, served as controls. A written informed consent was signed by all subjects before enrollment and the study was approved by the Ethical Committees, Faculty of Medicine, Alexandria University and Maadi Armed Forces Medical Compound.

Patients subjected to SCT (Group I and II) included 13 males (65%) and 7 females (35%) with an age range of 19-50 with a mean of 31.55±9.56 and a median of 32 years. Group III (the healthy control) included 5 males and 5 females with an age range of 25-45 with a mean of 33±8.41 and a median of 31 years.

Methods: All patients were subjected to the following:

1- Full history taking.

2- Complete clinical examination.

3- Radiological investigation (ultrasound, CT scans X-ray and when indicated, positron emission tomography (PET).

4- Laboratory investigations included: (A) Routine investigations (liver and kidney function tests, CBC [10]. (B) Bone marrow examination [10]. (C) Viral screening for hepatitis B virus (HBV) antigen, hepatitis C (HCV) antibody, cytomegalovirus (CMV) IgM, EBV and IgG [11]. (D) Blood cultures weekly or when there is fever or signs of sepsis. (E) Procalcitonin (PCT) assessment on days 5, 10 and 15 post transplant by chemiluminescence immunoassay (DIA-SORIN)-S.P.A. via crescentino snc-13040 Saluggia (VC)-Italy [11]. Procalcitonin level was considered positive if it exceeds the upper limit by 50%.

5- Pulmonary function testing and Echocardiography.

Statistical analysis: Data was statistically analyzed using SPSS (Statistical Package for the Social Sciences) version 20 for windows. Descriptive data were presented as range, mean±SD and frequencies. Mann-Whitney test was used to compare groups. Spearman correlation coefficient was used to test the relationship between various variables p-value ≤0.05 was considered significant.

RESULTS

Clinical and demographic data: Our cohort study included 6 patients with AML; 5 with NHL; 2 patients with CML in blastic crisis, Aplastic anemia and relapsed ALL each; and one patient with HD, relapsed HD and relapsed AML post autologous BMT each.

Viral screening: Six patients (30%) were negative for viral screening, 6 (30%) were CMV +ve, 4 (20%) were HBV +ve (they received lamivudine during the transplantation period) and 6 patients (30%) were HCV +ve (in two of them the transplantation was postponed for 6 months due to elevated liver enzymes and they were treated with interferon and ribavirin).

Complete blood counts: The blood count parameters at different time points are presented in Table (1). There was significant difference in Hb level, platelet counts and WBCs counts among the patients all over the period of follow-up (in days 5, 10 and 15 post transplantation) in comparison to day 0 (p<0.05).

The level of procalcitonin in control group and patients all over the period of follow-up is shown in Table (2).

All the 20 patients had positive serum procalcitonin during the period of hospitalization post transplantation. The different levels of procalcitonin were corresponding to the degree of sepsis. There was significant difference between the patients' results, in days 5, 10 and 15 post transplant, and control group (p=0.001, 0.001 and 0.0125 respectively).

At day 5, 11 patients were febrile and in all of them PCT levels were above the cut-off except one (patient no. 7) in whom level rose by day 10 and cultures were positive only for the central venous line. Three of these febrile patients failed to grow any positive culture (patients no. 8, 9, 1nd 14) compared to only one who failed to show rise of PCT level by day 5.

At day 10, all the 10 studied patients were febrile and this was also reflected by elevated

PCT levels in all except 2 patients (patients no. 2 and 8); in both fever dropped within 1-3 days. In one of these patients all cultures were negative and in the second, only urine culture was positive denoting limited infection.

Seven out of the 20 febrile patients remained culture negative compared to only 2 of 20 with PCT levels below the cut-off.

At day 15, All the 9 patients with persistent fever had PCT level above the cut-off except one (patients no. 15).

Culture results:

In patients with autologous SCT, there were 5 patients (50%) with negative and 5 (50%) with positive cultures; some of them had positive one type of culture and others had positive more than one type (blood, central venous catheter (CVC), sputum, urine, stool). In allogenic stem cell transplantation, there were 2 patients (20%) with negative and 8 (80%) with positive; some of them had positive results in more than one culture.

Type of organism: Twenty two bacterial isolates were detected with equal numbers of gram positive and gram negative, 11 each. The most common type of organism detected was Staphylococcus aureus (8 patients, 36.4%) followed by E coli (6 patients, 27.3%), Klebsiella Pneumonia (3 patients, 13.6%), Klebsiella Oxytoca and Staphylococcus Epidermidis (2 patients each, 9%) and lastly Enterobacter Cloacie (one patient, 4.5%).

Correlation between procalcitonin levels (ng/l), days of fever and culture results: Elevated procalcitonin was more sensitive and detected sepsis earlier than culture; it was positive in cases with negative cultures. All patients had positive procalcitonin, while only 5/10 with autologous and 8/10 with allogeneic transplantation had positive cultures. The types of positive cultures for the 20 patients are presented in Table (3).

Table (1): Peripheral blood counts of 20 transplanted patients at different time.

Parameter	Day 0	Day 5	Day 10	Day 15
Hb: g/dl	4.70-14.70*	5.30-11.50	5.30-10.30	5.10-9.30
	9.42±2.46	8.29±1.83	6.96±2.12	7.52±0.95
p		0.009	0.011*	0.029*
Platelets	9.00-196.00	7.0-69.00	4.00-61.00	4.00-181.00
x 10^{12}/L	84.70±54.80	26.05±17.04	17.21±14.68	56.06±51.51
p		0.001*	0.001*	0.05*
TLC	0.10-49.20	0.10-0.80	0.10-3.50	1.10-6.30
X 10^9/L	8.61±12.49	0.22±0.19	0.49±0.81	2.63±1.55
p		0.002*	0.004*	0.026*

Hb = Haemoglobin. TLC = Total Leukocytic count.

Table (2): Procalcitonin in 20 transplanted patients and control group at different time points.

Procalcitonin (µg/L)	Control	Day 5	Day 10	Day 15
Range	0.025-0.098	0.10-19.60	0.10-12.00	0.10-1.20
Mean±S.D.	0.0325±0.025	1.47±4.41	1.05±2.66	0.27±0.26
p		0.001*	0.001*	0.0125*

Table (3): Correlation between procalcitonin levels (ng/l), days of fever and culture results in 20 transplanted patients.

Patient / Parameter	Day 5	Day 10	Day 15	Days of fever	Culture results				
					Blood	Urine	CVC	Sputum	Stool
1	<0.1	0.43	0.24	$D_7 \rightarrow D_{15}$	(+ve)	–ve	+ve	–ve	–ve
2	0.10	0.13	0.12	$D_6 \rightarrow D_{13}$	–ve	(+ve)	–ve	–ve	–ve
3	0.52	0.71	0.2	$D_4 \rightarrow D_{15}$	(+ve)	–ve	(+ve)	–ve	–ve
4	0.41	0.9	0.3	$D_3 \rightarrow D_{16}$	–ve	–ve	(+ve)	–ve	–ve
5	0.15	12	1.2	$D_4 \rightarrow D_{17}$	(+ve)	–ve	(+ve)	–ve	–ve
6	0.11	0.18	<0.1	$D_{10} \rightarrow D_{13}$	–ve	–ve	–ve	–ve	–ve
7	<0.1	0.42	0.19	$D_4 \rightarrow D_{14}$	(+ve)	–ve	(+ve)	–ve	–ve
8	0.69	0.1	–	$D_4 \rightarrow D_{11}$	–ve	–ve	–ve	–ve	–ve
9	0.36	0.64	0.15	$D_4 \rightarrow D_{14}$	–ve	–ve	–ve	–ve	–ve
10	0.29	0.76	0.3	$D_5 \rightarrow D_{17}$	–ve	–ve	(+ve)	–ve	–ve
11	0.11	0.53	0.41	$D_7 \rightarrow D_{16}$	–ve	–ve	–ve	–ve	–ve
12	<0.1	0.16	0.5	$D_6 \rightarrow D_{17}$	–ve	–ve	–ve	(+ve)	–ve
13	<0.1	0.75	0.24	$D_8 \rightarrow D_{16}$	(+ve)	–ve	–ve	–ve	–ve
14	0.38	0.59	0.17	$D_4 \rightarrow D_{14}$	–ve	–ve	–ve	–ve	–ve
15	0.23	0.46	0.13	$D_2 \rightarrow D_{15}$	(+ve)	–ve	–ve	(+ve)	–ve
16	<0.1	0.36	0.1	$D_8 \rightarrow D_{13}$	(+ve)	(+ve)	–ve	–ve	–ve
17	5.2	0.28	0.16	$D_3 \rightarrow D_{14}$	(+ve)	–ve	(+ve)	–ve	–ve
18	19.6	–	–	D_7	–ve	–ve	–ve	–ve	–ve
19	0.67	0.34	0.13	$D_3 \rightarrow D_{13}$	(+ve)	–ve	–ve	(+ve)	–ve
20	0.12	0.21	0.14	$D_{76} \rightarrow D_{13}$	–ve	–ve	–ve	–ve	–ve

D: Day. CVC: Central venous catheter.

DISCUSSION

Infections contribute significantly to morbidity and mortality after hematopoietic stem cell transplantation (HSCT). Gram positive bacteria now account for 70% of bacterial infections compared with 30%, 25 years ago. This increase is in large part attributable to the nearly universal use of indwelling central venous catheters, and the wide spread use of antibiotics with gram-negative coverage to decontaminate the gut and reduce gram negative infections which has also contributed to the proliferation of gram-positive organisms **[13]**.

Boulad et al., (1998) found that infection is the result of a shift in the equilibrium between host defenses and microorganism pathogenecity. Patients undergoing HSCT are at risk of granulocytopenia, impairement of barrier defenses, and impairement of cell-mediated immunity and humoral immunity this will allowing microorganisms to cause infection more easily **[14]**.

Until recently no biomarker has been able to differentiate bacterial infection from a viral or non-infectious inflammatory reaction. Procalcitonin (PCT) is one of the first to offer this possibility **[15,16]**. Bacterial infections induce a ubiquitous increase of CALC-1 gene expression and a constitutive release of PCT from all parenchymal tissues, so that significant concentrations of PCT can be detected in the blood of patients with severe bacterial infection and sepsis **[17,18]**.

Procalcitonin increases 2-3 hours post induction by endotoxin levels then rises rapidly up to several hundreds in severe sepsis and septic shock. It remains high for up to 48 hours, falling to baseline values within the following 2 days **[19,20]**.

In our study, there was significant difference in the procalcitonin levels between patients in day 5, day 10, and day 15 due to the wide range detected. In all patients, the PCT results were positive, and reflecting the degree of septicemia in all patients.

PCT was positive with negative culture at early time points while with overt infection both PCT and cultures are positive.

This highlights the possibility that PCT levels may be better than cultures only for detecting infection early rather than late persistent infections.

In agreement with our results, PCT is superior to cultures in detection of sepsis with a sensitivity and specificity of 94 and (90%) at a cut-off of 0.5ng/1. Similar results were obtained in other studies **[21,22]**.

In our results, only 50% of patients with autologous and 80% of patients with allogeneic transplantation had positive cultures, while all patients had fever and had (except one) positive PCT achieving 90% sensitivity.

In clinical practice, the use of PCT as a diagnostic marker was shown to be of benefit in detection of sepsis. Because the up-regulation of PCT is attenuated by interferon-gamma, a cytokine released in response to viral infections, PCT is more specific for bacterial infections and may help to distinguish bacterial infections from viral illnesses.

In addition, PCT levels correlate with bacterial load and severity of infection which may help to guide the need for antimicrobial therapy more definitively **[23]**.

Among our patients, 13 (65%) had positive cultures compared to the serum PCT which was elevated in the vast majority of our patients with infectious fever.

This is in agreement with the results of other studies establishing the superior diagnostic accuracy of PCT in early infection compared with other markers **[24-26]**.

In conclusion, PCT is more sensitive and rapid to detect sepsis in the patients as the procalcitonin could detect sepsis in the cases with negative cultures and it was more correlated to fever than cultures in these patients.

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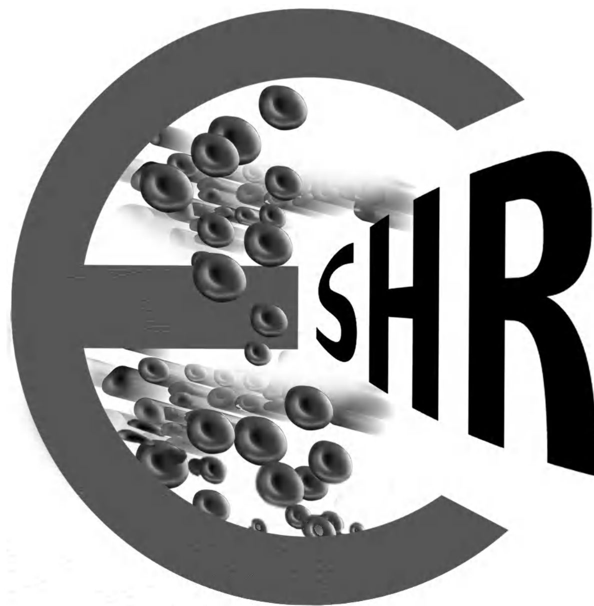

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Study of Some Apoptotic and Fibrotic Markers in Myeloproliferative Neoplasms

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ABSTRACT

Background: Chronic myeloproliferative neoplasms are clonal hematopoietic stem cell disorders characterized by proliferation, in the bone marrow, of one or more of the myeloid lineages. One of the important bone marrow findings that overlap the various CMPN entities is fibrosis.

Objectives: To evaluate the role of NF-k *ß* in patients with Chronic Myeloproliferative Neoplasms (MPB) in relation to some apoptotic (cathepsin, TGF *ß*) and fibrotic markers (b-FGF, BM reticulin score).

Patients and Methods: The study included 20 patients with de novo CML (group A) who received imatinib 400mg/day for 3 months; group B included 20 patients with other MPN [Essential thrombocytosis (ET), polythycemia vera (PV) and idiopathic myelofibrosis (IMF) who received hydroxy-urea orally in a dose of 0.5-2g/day for 3 months. Ten age and sex matched healthy individuals served as a control group. All patients were subjected to thorough history taking, full physical examination and laboratory investigations including CBC, BM biopsy and reticulin stain. NF-k *ß*, cathepsin B, TGF-*ß* and b-FGF were measured using ELISA technique.

Results: The mean values of NF-k *ß* in CML patients (group A) and non CML-CMPN (group B) were 165.20± 26.96 and 200.79±92.00pg/ml respectively while after treatment the values were significantly decreased to 104± 25.36 and 134.2±96.89pg/ml respectively (*p*=0.013, *p*= 0.001). Cathepsin B increased significantly in group A after 3 months of therapy (From 71.90±39.85 to 126.80± 60.42pg/ml in CML, while in group 2 it decreased significantly from 123.47±50.67 to 67.50±42.50pg/ml (*p*=0.001 and 0.001 respectively). The level of TGF- *ß* decreased in CML from 101.20±38.16 to 52.20±22.06 while in group 2 it decreased from 128.53±63.54 to 87.45±48.87pg/ml (*p*=0.001) b-FGF level also decreased after treatment; in CML it decreased from 14.68±4.33 to 11.03±3.22 (*p*=0.042), while in group 2 it decreased from 35.65±9.85 to 15.52±3.2pg/ml (*p*=0.001). Reticulin stain score of BM biopsy showed significant decrease of BM fibrosis in group A and B (*p*=0.036 and 0.0001 respectively).

Conclusion: NF-κ *ß*, TGF-*ß* and Cathepsin B are beneficial in monitoring the disease progression and response to treatment in myeloproliferative neoplasms. These data support further use of these markers in the prognosis of CMPN.

Key Words: *CMPN = Chronic Myeloproliferative Neoplasms – NFK-b = Nuclear Kappa B cell – b-FGF = basic Fibroblast Gowth Factor – TGF-B = Transforming – Growth Factor Beta.*

INTRODUCTION

Chronic myeloproliferative neoplasms (CMPN) are clonal hematopoietic stem cell disorders characterized by proliferation, in the bone marrow, of one or more of the myeloid lineages [1]. One of the important bone marrow findings that overlap the various CMPN entities is fibrosis which is most likely caused by abnormal production of platelet derived growth factor (PDGF) and transforming growth factor-*ß* (TGF- *ß*) by Megakaryocytes [2].

The caspases are a family of proteins that are one of the main executors of the apoptotic process [3,4]. Nuclear factor-kappa *ß* (NF-k*ß*) is a protein that acts as a switch to turn inflammation on and off in the body. NF-k*ß* acts in each of the main phases of cancer development [5]. NF-k*ß* activation in inflammatory cells results in increased production of cytokines and other growth factors that support the growth, replication and invasion of cancerous cells. Such activated inflammatory cells provide new blood vessel formation [5-7].

b-FGF is an endothelial cells growth factor. It is an important inducer of stromal cell activation; activated stromal cells, in turn, may

produce other inducers of angiogenesis such as b-FGF, IL-6 and IL-8 [8].

TGF- *ß* regulates cellular proliferation in a cell specific manner. In most endothelial and hematopoietic cells, TGF- *ß* is a potent inhibitor of cell proliferation. In cancer cells, mutations in the TGF- *ß* pathway have been described as it allows uncontrolled proliferation of the cells [6-10]. In response to increased production of TGF- *ß* by the tumor cells, they become more invasive and metastasize to distant organs [9,11,12].

TGF-*ß* increases the biosynthesis of type I, III, IV collagens, fibronectin and proteoglycans. It blocks matrix degradation and acts as a potent angiogenic factor [13].

Cathepsins are proteases found in many types of cells. Cathepsin B can participate in tumor invasion by degradation of extracellular matrix components. Cathepsin B enhances apoptotic pathways [14-17].

In this study, we aimed to evaluate the role of NF-k*ß* in patients with chronic myeloproliferative meoplasms (CMPN) in relation to some apoptotic (cathepsin, TGFB) and fibrotic markers (b-FGF, BM reticulin stain score).

PATIENTS AND METHODS

Patients: The study included 40 CMPN patients admitted to the Hematology Department, Main Alexandria University Hospital. They were divided into two groups: Group A comprised 20 (50%) de novo CML patients; they received imatinib 400mg/day for 3 months and group B comprised 20 patients with other MPN-ET 8 (20%), PV 5 (12.5%) and IMF7 (17.5%); they received 0.5gr-2gr/day oral hydroxyurea for 3 months. Three patients did not return back for follow-up in group B (missed). Ten healthy subjects with matched age and sex were a control group. The study was approved by the Institutional Review Board and a written informed consent was obtained from all subjects before enrollment.

Methods: All patients were subjected to complete blood count [18], Renal and Liver profile [19], BM biopsy using hematoxylin and eosin and reticulin stain [20], PCR for BCR-ABL [21] and JAK2 V617F [22], measurement of NF-Kb (NF-κB Transcription Factor Assay Kit, Rockland Immunochemicals, USA), Cathepsin B (Abcam's Human Cathepsin B in vitro ELISA, USA), TGF-*ß* (Human TGF-beta 2 Quantikine ELISA Kit R&D Systems), b-FGF (Human FGF basic Quantikine HS ELISA Kit-R&D system) [23].

RESULTS

Demographic data: The age range of group A was 22-67 with a mean of 44.85±14.78 and a median of 50 years; for group B the range was 20-75 with a mean of 48.45±11.13 and a median of 51 years; and for the control, the range was 26-62 with a mean of 45.70±14.77 and a median of 42 years. The patients included 16 males (40%) and 24 (60%) females; the control included 4 males (40%) and 6 (60%) females. The patients included 20 (50%) cases with de Novo CML in chronic phase (Group A). Group B included 8 (20%) patients with essential thrombocythemia, 7 (17.5%) with idiopathic myelofibrosis and 5 (12.5%) with polycythemia vera.

Peripheral blood counts (Table 1): There was significant decrease in platelet counts as well as in WBCs in group A and B after treatment (p=0.001). In group A Hb level increased after therapy but not significantly.

Bone marrow cellularity: Regarding group A, BM was hypercellular in all 20 (100%) patients. The hypercellularity was secondary to myeloid metaplasia before therapy; after therapy with imatinib, all patients exhibited a decrease in cellularity. The nadir cellularity in 12 patients was normocellular to moderately hypocellular. The reduction in cellularity corresponded to prominent decrease in myeloid proliferation in all patients with the most severe reduction in cellularity characterized by decrease in both myeloid and erythroid production. The number of megakaryocytes remained normal in all except the 8 patients with marked hypocellularity, in whom the number was decreased.

Group B: Before therapy there were 4 patients (20%) with normocellular, 3 (15%) with hypocellular, and 13 (65%) with hypercellular BM; after treatment BM cellularity returned to normocellular in 9 patients (52.9%), 1 (5.9%)

remained hypocellular, 7 (41.2%) were hypercellular while 3 patients refused to repeat the trephine. There was significant improvement in BM cellularity after therapy (p=0.0001). Erythroid hyperplasia was prominent in PV and moderately to markedly reduced in IMF. Granulopoiesis showed a relevant increase in PV, but normal granulopoiesis in ET. In PV there was increase in clustered enlarged megakaryocytes with hyperploid nuclei.

NF-k ß, Cathepsin B, TGF-ß, b-FGF and Reticulin stain score before and after treatment (Table 2):

In patients with CML (group A) and ET, PV and IMF (group B) the level of NF-kß both before and after treatment was increased in relation to the control group (p=0.001), but there was significant decrease of NF-kß after treatment in both groups (p=0.013, p=0.001 respectively). Cathepsin B increased significantly in group A and B after 3 months of therapy (p=0.001 and 0.001 respectively). Also the level of TGF-ß declined significantly in both groups (p=0.001and 0.001). Regarding b-FGF it decreased significantly in both groups (p=0.013, 0.001). Reticulin stain score of BM biopsy (Figs. 1, 2, 3 and 4) showed significant decrease in BM fibrosis after treatment in both groups (p=0.036 and 0.0001 respectively).

Correlation of NF-k ß, Cathepsin B, TGF-ß, b-FGF and Reticulin score with other parameters (Table 3):

There was significant positive correlation between NF-k ß and TGF- ß, b-FGF and Reticulin score, Hb, platelets and bone marrow Cellularity but no significant correlation with other parameters (RBC, erythopoiesis, Myelopoeisis , megakaryopoiesis and blast cells).

Table (1): Peripheral Blood parameters in 40 chronic myeloproliferaive patients before and after treatment.

Parameter	CML patients		Non-CML MPNs Patients		Control
	Before treatment	After treatment	Before treatment	After treatment	
WBCx10 ^{9}/L:					
Range	45.62-235.25	7.5-22.1	4.53-39.3	4.5-23	3.9-10.4
Mean±S.D.	182.11±65.11	15.89±4.90	16.68±10.36	10.39±5.17	7.51±2.27
p	0.0001*	0.0001*	0.001*	0.013*	
p_1	0.001*		0.001*		
RBCx10 ^{12}/L:					
Range	3.32-4.71	3.63-5.14	3.1-8.91	2.35-7.27	3.97-5.3
Mean±S.D.	3.99±0.23	4.22 ±1.01	5.13±1.90	5.20±1.13	4.85±0.47
p	0.233	0.365	0.412	0.365	
p_1	0.096		0.254		
Hb gm/dl:					
Range	8.8-10.20	7.1-11.3	6.9- 20	6.9-18	11.1-14.5
Mean±S.D.	9.18±2.05	10.16±2.43	11.95±3.91	12.65±2.62	12.68±1.62
p	0.022*	0.045*	0.265	0.652	
p_1	0.265		0.18		
HCT %:					
Range	27.9-46.1	22.9-42.7	23.8-57.4	22.6-53.0	35.1-42.2
Mean±S.D.	32.11±4.62	30.91±6.04	38.0±12.0	39.0±9.0	39.18±2.30
p	0.132	0.098	0.468	0.852	
p_1	0.236		0.336		
PLTx10^9/L:					
Range	198-550	216-445	110-1985	159-765	177-368
Mean±S.D.	365.11±76.28	327.11±81.94	766.45±588.7	356.20±155.65	274.2±77.76
p	0.016*	0.098	0.001*	0.089	
p_1	0.136		0.001*		

p : Comparison between control and other groups.
p_1 : Comparison between before and after treatment in the same group.

Table (2): NF-k β, Cathepsin B, TGF- β, b-FGF and Reticulin score before and after treatment in CML patients and Non-CML MPNs compared to control.

	CML Patient No=20		Non-CML MPNs Patients: No=20		Control No=10
	Before treatment	After treatment	Before treatment	After treatment	
NF-k β: pg/ml:					
Range	39-233	33-179	60-359	35-390	20-65
Mean±S.D.	165.20±26.96	104.0±25.36	200.79±92.0	134.2±96.89	45.2±16.57
p	0.001*	0.001*	0.001*	0.001*	
p_1	0.013*		0.001*		
Cathepsin B: (pg/ml):					
Range	28-137	17-198	11-200	10- 240	21-123
Mean±S.D.	71.9±39.85	126.80±60.42	123.47±50.6	67.50±42.50	69.4±41.41
p	0.236	0.001*	0.001*	0.685	
p_1	0.001*		0.001*		
TGF-β (pg/ml):					
Range	30-174	18- 83	10-222	7-188	10-28
Mean±S.D.	101.2±38.16	52.20±22.06	128.53±63.54	87.45±48.87	17.9±7.17
p	0.001*	0.001*	0.001*	0.001*	
p_1	0.001*		0.001*		
bFGF (pg/ml):					
Range	6.5-18	7-14	18-68	9-20	8-15
Mean±S.D.	14.68 ±4.33	11.03±3.22	35.65±9.85	15.52 ±3.21	11.25±2.98
p	0.042*	0.526	0.001*	0.107	
p_1	0.013*		0.001*		
Reticulin Stain score:					
Min-Max.	0-2	0-1	2-4	1-3	
Mean±S.D.	1.42±0.31 p=0.036*	0.561±0.12	3.05±0.76 p=0.001*	2.00±0.79	

p = Comparison before and after treatment.
p_1 = Comparison between group A and group B.

Table (3): Correlations between different studied parameters in all patients.

		NF-k β	Cathepsin	TGF- β	b-FGF	Reticulin stain score
NF-kB (pg/ml)	*r*		0.477**	0.711**	0.404**	0.644**
	p		0.001	0.001	0.006	0.001
Cathepsin B (pg/ml)	*r*	0.509**		0.807**	0.622**	0.787**
	p	0.001		0.001	0.001	0.001
TGF-B (pg/ml)	*r*	0.699**	0.898**		0.455**	0.791**
	p	0.001	0.001		0.002	0.001
b-FGF (pg/ml)	*r*	.401**	.455**	.442**		.347*
	p	0.006	0.002	0.002		0.019
Reticulin stain score	*r*	.327*	.601**	.615**	.298*	
	p	0.028	0.001	0.001	0.047	
RBC	*r*	0.234	0.099	0.112	0.29	0.279
	p	0.122	0.52	0.465	0.053	0.063
HGB	*r*	.455**	0.064	.349*	0.182	0.208
	p	0.002	0.677	0.019	0.232	0.17
PLT	*r*	.381*	.475**	0.143	.412**	0.243
	p	0.011	0.001	0.348	0.005	0.108
BM Cellularity	*r*	.307*	.470**	.631**	0.205	.435**
	p	0.04	0.001	0.001	0.176	0.003
Erythropoiesis	*r*	0.283	0.132	0.178	0.174	0.254
	p	0.059	0.387	0.241	0.252	0.093
Myelopoeisis	*r*	0.122	0.276	0.262	0.164	0.247
	p	0.201	0.066	0.083	0.282	0.102
Megakaryopoiesis	*r*	0.174	.344*	0.234	0.099	0.112
	p	0.23	0.021	0.122	0.52	0.465
BLASTS	*r*	0.185	–0.087	–0.14	0.168	–0.099
	p	0.225	0.571	0.361	0.271	0.516

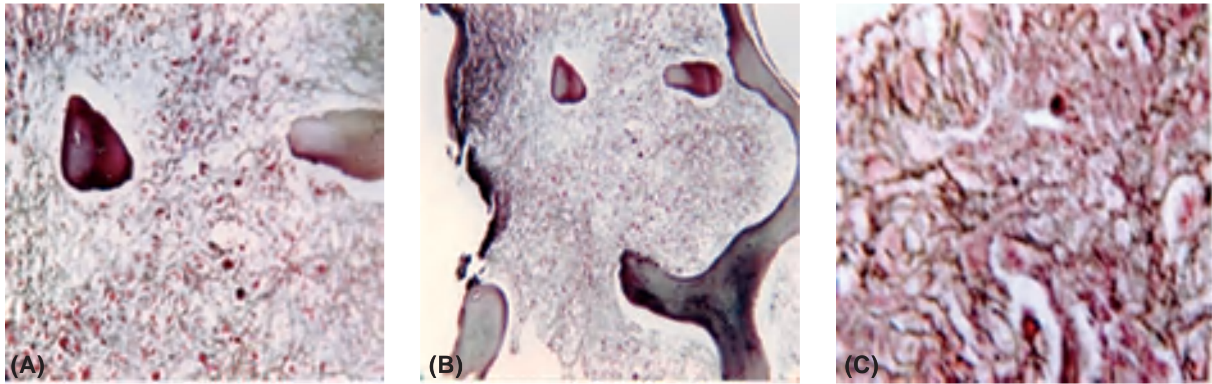

Fig. (1-A,B,C IMF): Grade 1 BM fibrosis: Reticulin stain showing normal scattered fine fibers (200x magnification).

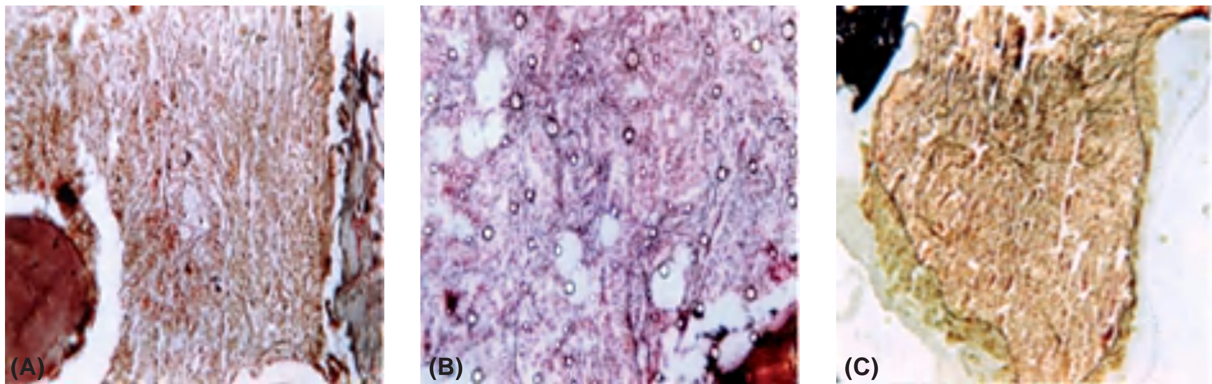

Fig. (2-A,B,C ET): Grade 2 BM fibrosis: Reticulin stain showing a fine network of fibers with no coarse fibers (200x magnification).

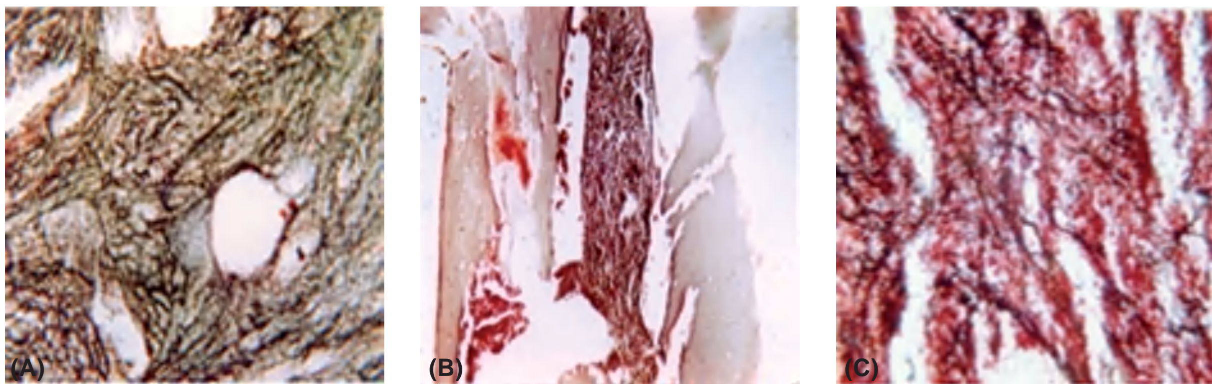

Fig. (3-A,B,C CML): Grade 3 BM fibrosis: Reticulin stain showing diffuse fine reticulin and scattered thick coarse fibers, with no mature collagen (200x magnification).

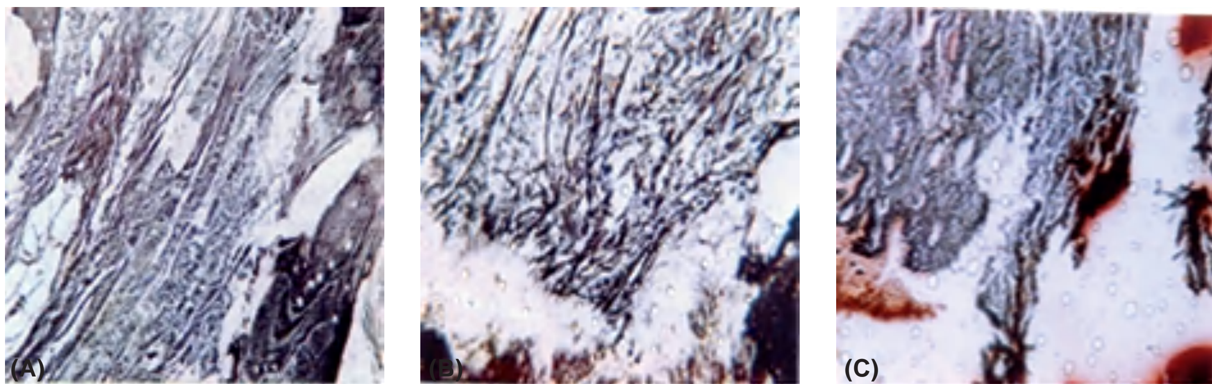

Fig. (4-A,B,C PV): Grade 4 BM fibrosis: Reticulin stain showing coarse reticular fiber network, with collagen deposition (200x magnification).

DISCUSSION

Little is known about the signal transduction pathways involved in the development of CMP-Ns. However, an activation of the JAK/STAT pathway is involved in myeloproliferative disorders and BCR-Abl tyrosine kinase in CML for which the molecular mechanisms have been characterized [17]. In the present study, we showed that other signal transduction pathways, NF-k*ß*, TGF-*ß*, cathepsin B, and b-FGF were also activated.

Kidney functions improved after treatment in both groups. The improvement was significant for creatinine in both patients group A & B, before treatment it was 1.89 and 1.11mg/dl while after treatment the mean value was 1.35 and 0.69mg/dl respectively. Comparing these values in the patient groups before and after treatment, a statistically significant differences were found (p=0.036, 0.01). Plomely et al., [24] reposted a case of PV which was found to have a high blood urea nitrogen and serum creatinine. The patient was initially subjected to phlebotomies and showed symptomatic improvement and serum creatinine decreased with the normalization of its level.

Regarding CML patients, bone marrow was hypercellular in all 20 patients (100%) before treatment, after 3 months therapy with imatinib, all patients exhibited a decrease in cellularity and decreased myelopoiesis. These observations are in agreement with Hasserijian et al., [25] who concluded that reduction in marrow cellularity in response to imatinib was independent of cytogenetic response. However John et al. found that longer follow-up of the histopathologic features of bone marrow biopsy specimens showed striking differences between patients who remained positive or negative for BCR-Abl [26].

Reticulin stain score of BM biopsy showed significant decrease in fibrosis. A reduction in marrow fibrosis in CML cases treated with imatinib mesylate has been reported [27] even in cases in which the malignant clone is not eradicated.

In our cohort, 65% of non CML MPN (group B) had hypercellular BM before treatment, 15% hypocellular and 20% monocellular, while after treatment 41.2% had hypercellular and 59% normocellular BM. Our results are in agreement with Thiele et al., who reported significant decrease in erythroid precursors in CML compared to control, while this cell lineage was most prominent in PV and moderately to markedly reduced in IMF with clusters of small to giant sized megakaryocytes in PV. In IMF more than 80% of patients present with some degree of myelofibrosis-osteosclerosis at diagnosis, while the rest show an initial prefibrotic, hypercellular stage [28].

In this study, the values of NF-k*ß* decreased significantly after treatment in both CML and non CML patients (p=0.013, p=0.001). Guicciardi et al., [15] reported that NF-k*ß* activates multiple target genes whose products can block the apoptotic program triggered by death receptors or the mitochondrial pathway; NF-k*ß* inducible anti-apoptotic factors include those that inhibit caspase function and those that inhibit NF-k*ß* signaling after TNF-R1 stimulation. Stimulation of TNF-R1 induces the breakdown of the lysosome and the induction of apoptosis by cathepsins released into the cytoplasm.

In the current study, cathepsin B level was significantly increased in all patients (p=0.001) after treatment. Foghogaard et al., [14] stated that several observations may lead to conclude that the induction of serine protease inhibitor (SPi2A) by NF-k*ß* protects cells from TNF-alpha by inhibiting the lysosomal pathway of apoptosis. Inhibition of cathepsin B protects NF-k*ß* from TNF-alpha induced apoptosis, confirming the observation that cathepsin B plays a direct role in apoptosis in other cell types. Scaffidi et al., [29] suggested that after ligation of TNF-R1, Spi2A inhibits cytoplasmic cathepsin B activity and so may prevent apoptosis by inhibiting the cleavage of Bid by cathepsin B and subsequent activation by proteins released from mitochondria.

The increase in cathepsin B after therapy in our study agrees with the previous studies as its increase denotes caspase activation [30]. Cathepsin B is a potent inducer of apoptosis and is released into the cytoplasm where it activates caspase dependent and caspase-independent pathways of cell death.

In this study, we reported that the level of TGF-*ß* was significantly decreased after treatment in both patient's groups (p=0.001). This

is in agreement with others who found that an abnormal release of growth factors such as TGF-*β* could be responsible for the development of marrow fibrosis **[30]** and that activation of NF-k *β* leads to increase of TGF- *β* expression **[31,32]**.

In non CML patients the mean value of reticulin stain score of BM biopsy had markedly decreased after treatment (p=0.001) raising the possibility that fibrosis has decreased due to the inhibitory effect of NF-k *β* on TGF- *β*.

In our patients the level of b-FGF was significantly higher in non CML in comparison with CML patients; it significantly decreased in both groups post treatment (p=0.001, 0.013). Increased plasma levels of b-FGF have also been reported by others in patients with IMF, ET and PRV **[33,34]**.

There was significant positive correlation between NF-k *β* and TGF- *β*, b-FGF and Reticulin score, Hb, platelets and bone marrow Cellularity but no significant correlation with other parameters (RBCs, erythopoiesis, Myelopoeisis , megakaryopoiesis and blast cells). To the best of our knowledge, such correlations have not been previously addressed. Our results might indicate that the inhibition of NF-k*β* by treatment leads to the activation of the apoptotic pathway and that the release of cathepsin B is a sign of apoptosis progression. The NF-κ*β* inhibits TGF-*β* and b-FGF which are markers of fibrosis.

Conclusion:

The use of NF-κ*β*, TGF-*β* and Cathepsin B is beneficial in monitoring the disease progression and response to treatment in myeloproliferative neoplasm. These data support further use of these markers in monitoring the prognosis of CMPN. More prospective randomized controlled trials and large sample size are still required to precisely understand the role of NF-κ*β*, TGF-*β* and Cathepsin B in the pathology of CMPN. Implementation of the use of NF-κ *β*, TGF-*β* and Cathepsin B as prognostic markers of CMPN is strongly recommended.

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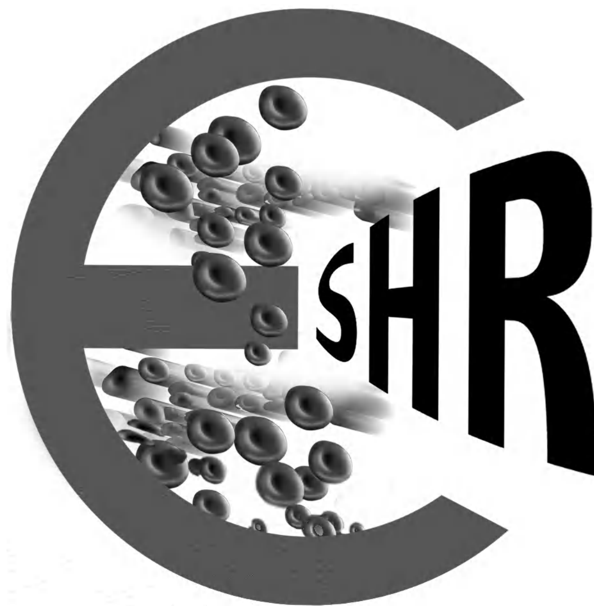

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Telomerase Enzyme Activity in Egyptian Children with Bone Marrow Failure and Response to Immunsupressive Therapy

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ABSTRACT

Background: The development of bone marrow failure might be related to low telomerase activity (TA).

Objectives: We aimed to evaluate TA in children with inherited bone marrow failure (IBMF) and acquired aplastic anemia (AAA). The relation of the acquired disease to TA and response to immunosuppressive therapy (IST) were studied.

Patients and Methods: TA in mononuclear cells was measured utilizing Telomeric Repeat Amplification Protocol (TRAP) in 40 patients and 40 controls.

Results: Median TA was lower in IBMF patients compared to controls (p=0.04), for the AAA cases it was comparable to controls (p=0.228). There was an inverse correlation between TA and age (r=–0.39, p=0.026) but not with disease duration (r=–0.33, p=0.111). Twenty seven AAA patients received Cyclosprine A (CSA); 19 (70.4%) were responderswith median TA of 16.5±4.7 vs. 11.6±3.8 for none responders (p=0.6). Area under the curve (AUC) of TA in predicting IST response among AAA cases was 0.569 (p=0.540).

Conclusion: TA was low in 90% of IBMF and in 23% of severe AAA patients. Evaluation of TA might not be essential for therapeutic or prognostic aspects of AAA. However, it might be useful for selection of stem cell family donors in AAA and IBMF patients with low telomerase activity.

Key Words: *Bone marrow failure – Immune therapy – Telomerase activity – Aquired aplastic anemia.*

INTRODUCTION

The most common cause of bone marrow failure is acquired aplastic anemia (AAA). The inherited bone marrow failure syndromes include Fanconi anemia, dyskeratosis congenita, Diamond-Blackfan anemia, and other genetic disorders. Fanconi's anemia and dyskeratosis congenita are the most common types of constitutional aplastic anemia [1]. Patients with constitutional aplastic anemia were found to have strikingly short telomeres and low telomerase activity in their cells [2]. Telomeres are structural elements that seal the ends of chromosomes, protecting them from recombination, end-to-end fusion, and recognition as damaged DNA. Telomere erosion has been associated with the process of normal aging and defective telomere maintenance is a feature of a variety of human diseases including constitutional aplastic anemia [3,4]. Maintenance of the integrity of telomeres requires the telomerase ribonucleoprotein complex [5-8]. Most of the acquired aplastic anemia (AAA) is the result of an immune process that destroys hematopoietic stem and progenitor cells [9,10]. It was assumed that the predisposition to the development of acquired marrow failure appears to be conferred by genetic alterations resulting in low telomerase activity, short telomeres in leukocytes, and reduced hematopoietic function [11,12]. Several studies reported short telomeres and low telomerase activity in leukocytes in up to one third of patients with AAA, especially those who were resistant to immunosuppressive therapy [12-15]. Most of the previous studies were concerned mainly with the detection of telomere length and the mutant genes causing low telomerase activity rather than telomerase activity [12-15].

In this study we aimed primarily to evaluate the telomerase functional activity in Egyptian children with inherited bone marrow failure (IBMF) and acquired bone marrow failure namely acquired severe aplastic anemia (AAA).

The relation of the acquired disease to telomerase enzyme activity and response to immunosuppressive therapy were also studied.

PATIENTS AND METHODS

This was a case-control study conducted on unrelated children (n=40) with bone marrow failure syndromes attending the Hematology Clinic during the study period over 3 months from February to April 2014 and forty healthy subjects (age- and sex-matched) taken as controls. The diagnosis of bone marrow failure was based on the presenting clinical features, the blood-count and bone marrow biopsy criteria of the International Agranulocytosis and Aplastic Anemia Study [16]. Both groups were enrolled in the study after obtaining consents from their legal guardians and the approval by the Ethical Committee of Cairo University.

Patients included 23 (57.5%) males and 17 (42.5%) females with M/F ratio of 1.35. Mean age of the studied cases was 11.1±4.9 years (range 3.5 to 18, median 11 years). Patients' records were thoroughly reviewed and detailed history-taking was carried out. Disease severity at presentation as well as lines of management and response to immunosuppressive therapy was recorded. Among AAA patients, the disease was considered severe if at least 2 of the following criteria were noted: Neutrophil count less than $0.5x10^9$/L; platelet count less than $20x10^9$/L with hypocellular bone marrow [17].

Immunosuppressive regimens: In AAA patients (n=30); 27 cases received cyclosporine A (CSA) as a monotherapy in a dose range of 7 to 10mg/kg/dto maintain CSA levels between 200-400ng/mL. A combination of anti-thymocyte globulin (ATG) and CSA were given in 3 patients; ATG as a single course for 5 days as intravenous infusion over 12 to 18 hours through a central venous catheter and oral CSA at 5mg/kg/day with the ATG. Oral steroids at a dose of 1mg/kg, prior to each daily dose of ATG to prevent serum sicknesstapered slowly over 4 weeks, was also given. Response to IST was evaluated after initiation of therapy for 3 to 6 months and or follow-up duration ranging from 1 to 13 years with a mean of 5.14±3.84 years.

Evaluation of IST response: Complete response (CR) was defined as a neutrophil count more than $1.5x10^9$/L, a platelet count more than $100x10^9$/L, and a hemoglobin level more than 11.0g/dL [18]. Partial response (PR) if neutrophil count was more than $0.5x10^9$/L, platelet count more than $20x10^9$/L, and hemoglobin level was more than 8.0g/dL [18].

Telomerase activity study: Peripheral blood samples were collected from patients and controls under aseptic technique. Mononuclear Cells were separated by Ficoll-Hypaque density gradient centrifugation [19,20] and the level of telomerase activity was accurately measured utilizing the Telomeric Repeat Amplification Protocol (TRAP) using the TeloTAGGG Telomerase PCR ELISA^{PLUS} according to Quach et al., [21].

Statistical analysis: Data management and analysis were performed using SigmaStat program; version 3.5 (Systat Software, Inc., USA). The numerical data were statistically presented in terms of range, mean, standard deviation, median and interquartile range (IQR). Categorical data were summarized as percentages. Comparisons between numerical variables of two groups were done by unpaired Student's *t*-test for parametric data or Mann-Whitney Rank Sum test for non-parametric data. Comparing categorical variables were done by Chi-square test or Fisher exact test for small sample size. Pearson product moment correlation test was used for correlating quantitative variables. Receiver's operating characteristics (ROC) curve was made and area under the curve (AUC) was calculated for the telomerase activity in predicting IST response. All *p*-values were two tailed and considered significant when *p*-values less than 0.05.

RESULTS

*Demographics and clinical data:*The study included 40 patients, 30 cases with severe AAA and 10 with IBMF. Mean age of AAA patients was 10.6±5.0 years (range 3.5 to 18 years, median 9.5yrs) and mean age at diagnosis was 5.2±2.9 (range 1-12, median 5 years). Patients with IBMF included 6 Fanconi anemia (FA), 1 constitutional AA, 1 Dyskeratosis congenita (DKC) and 2 with pure red cell aplasia (PRCA). Their mean age was 14.1±7.1 years (range 4 to 18 years, median 14yrs) and mean age at diagnosis was 6.2±4.3 (range 1.5-12, median 5 years).

Telomerase activity evaluation:

The median telomerase activity was significantly lower in patients with IBMF (p=0.04) while it was insignificantly lower incases with AAA compared to that of controls (Table 1). There was an inverse correlation between the telomerase activity and age (r=–0.39, p=0.026) but no correlation was found between the telomerase activity and disease duration (r=–0.33, p=0.111). Comparing with the mean TA of the control; 90% (9/10) of cases with IBMF had low telomerase activity versus 23% (7/30) of AAA patients (p<0.001).

Table (1): Telomerase activity of the study subgroups and control.

Telomerase level	Median (IQR[a])	Range	p-value
IBMFS[b] (n=10)	5.0 (4.6-8.7)	0.5-45.6	0.043[d]
Control (n=40)	11.2 (5.9-16.6)	1.2-39.0	
AAA[c] (n=30)	5.4 (2.3-21.0)	0.5-65.4	0.228
Control (n=40)	11.2 (5.9-16.6)	1.2-39.0	
IBMFS[b] (n=10)	5.0 (4.6-8.70)	0.5-45.6	0.851
AAA[c] (n=30)	5.4 (2.3-21.0)	0.5-65.4	

[a] IQR : Interquartile range.
[b] IBMFS : Inherited bone marrow failure syndrome.
[c] AAA : Acquired aplastic anemia.
[d] : Statistically significant.

Telomerase activity and response to IST:

All patients with AAA received immunosuppressive therapy for at least 6 months in the form of cyclosporine A (CSA) as a monotherapy (n=27) or combined with ATG (n=3). Nineteen out of the twenty-seven patients (70.4%) were responders to CSA (12 partial responders and 7 complete responders) with no responders among the three cases who received ATG. The median telomerase activity was 16.5±4.7 among AAA responders vs. 11.6±3.8 of none responders but the difference was not significant (p=0.6). Three of the seven cases with low TA responded partially or completely to CSA therapy while 70% (16/23) with normal TA responded to IST (p=0.2). We evaluated the sensitivity and specificity of telomerase activity in predicting the response of AAA patients to IST at different cut off values by ROC curve, we found that area under the curve of telomerase was 0.569 (95% CI 0.377 to 0.748; p=0.540) indicating that the overall predictability of telomerase activity is not significant. On fixing the sensitivity or specificity of telomerase activity, we found that either its sensitivity or specificity became unsatisfactory making its adoption as a good predictor of response in AAA to IST was unlikely (Fig. 1).

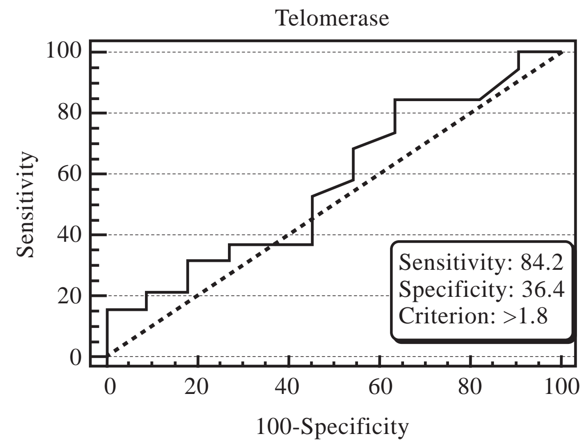

Fig. (1): Receiver Operating (ROC) curve of telomerase activity for prediction of response to Immunesuppressive therapy among acquired aplastic anemia subjects.

DISCUSSION

Our data showed that telomerase activity (TA) was detectable in all of our studied cases including healthy controls. Previous studies reported undetectable TA among healthy controls **[22]**. Children with IBMF had significantly lower TA compared to controls. In AAA patients, the median TA was comparable to IBMF cases; this might be explained by the small number of patients included in this subgroup. In this study up to 23% of cases with AAA had below normal TA which decreased significantly with theincreasing of age, but not related to disease duration. This study showed absence of asignificant direct relationship between TA and response to IST; its adoption as a therapeutic or prognostic predictor was unlikely.

Previous studies evaluated the telomere length and mutations that might affect TA rather than testing for TA directly in patients with acquired marrow failure with short telomeres **[4,11,12,14,23]**. Few authors evaluated TA in patients with AAA who had the TERC or TERT mutations and reported low activity. They found that subjects with low TA did not respond adequately to immunosuppressive therapy. However, their data were mainly descriptive due to the small sample size of patients they studied **[4,12]**.

In IBMF, our data was consistent with the previous studies that reported a low telomerase activity in these patients [2,24]. A recent study, which measured telomere length and telomerase activity in 71 aplastic anemia patients, reported that TA had no significant difference in terms of age or gender in the IST responders, non responders or control group. They also found that TA in severe and mild groups was significantly higher than normal control group [25]. These data were not in accordance with our data where 23% of our patients with severe AAA had low TA.

Our study had some limitations as we did not test for telomere length and gene mutations in the peripheral leukocytes of those with decreased telomerase activity. However, telomerase activity measurement using the TRAP assay may be an easy and highly sensitive method that may replace mutation and telomere length in the selection of suitable hematopoietic stem cell family donors for transplantation in patients with telomerase deficiency [21,26].

In conclusion, telomerase activity was low in up to 90% of hereditary BMF and in 23% of AAA patients. Evaluation of telomerase activity might not be essential for therapeutic or prognostic aspects of acquired aplastic anemia patients. However, it might be useful for selection of stem cell family donors in patients with acquired aplastic anemia and telomerase deficiency.

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