Journal of the Egyptian Society of Haematology & Research, Vol. 2, No. 2, September: 169-178, 2006

Evolution of the Accelerated and Blastic Phases of Chronic Myeloid Leukemia: Molecular, Cytogenetic, Flowcytometric and Electron Microscopic Studies

AMIRA M. KHORSHED, M.D.; AMIRA H. SOLIMAN, M.D.; NAHED ABDEL WAHAB, M.D.; SHERINE IBRAHIM, M.D.; KHALED A. EMARA, M.D.; REHAM RASHED, M.D.; TAREK MANSOUR, M.D.*; NAGLAA KHOLOUSY, M.D.** and FAYEK M. GHALEB, M.D. ***

The Departments of Clinical Pathology and Cancer Biology, National Cancer Institute, Cairo University and Immunogenetic, National Research Center** and Clinical Pathology, Ophthalmology Research Institute***.*

ABSTRACT

Background: Chronic Myeloid Leukemia is a clonal disease that results from an acquired genetic change in a pluripotential hemopoietic stem cell. Molecular abnormalities and mutations usually accompany the accelerated and blastic crisis phases of CML.

Aim of Work: This study was conducted to explore the possible ultrastructural, molecular cytogenetic, apoptotic and morphological abnormalities that may contribute to the progression of chronic phase to accelerated and blastic crisis phases in CML patients, for focusing on high risk patients to justify accurate lines of treatment.

Subjects and Methods: The study included thirty CML patients newly diagnosed and under treatment presenting to the Medical Oncology department of the National Cancer Institute, Cairo University, and ten age-matched subjects as a control group. CD95 (FAS) and p53 were studied by flowcytometry, BCR/ABL gene was studied at the cytogenetic and molecular level by RT PCR and ultrastructural apoptotic changes were studied by EM in PB samples. Also FISH was performed on few selected cases where conventional cytogenetics was not informative.

Results: Mean level of p53% was highly increased in the accelerated and/or blastic crisis phase and the chronic phase compared with controls (p=0.04). Mean level of CD95% expression was higher when measured on the whole cell population in the (accelerated and/or blastic crisis) compared with chronic phase and controls (p=0.14). By selecting CD34+ve cells, lower levels of CD95% expression were found in the (accelerated and/or blastic crisis phase) compared with the levels expressed on the whole cell population in the same phase. Cases were divided according to follow up into Group 1: 16/30 (53.3%) chronic phase cases that remained chronic during treatment. Group 2: 12/30 (40%) chronic phase cases that developed an accelerated or blastic crisis during treatment then returned to the chronic phase. Group 3: 2/30 (6.6%) chronic phase cases that developed an accelerated or blastic crisis phase then died. Mean p53% levels showed no statistically significant difference between the groups of CML (p=0.85). Higher levels of CD95% in CD34+ve cells were expressed in Group 1 compared with Group 2 and Group 3 and were statistically insignificant (p=0.85). Mean levels of CD95% expression were higher when measured on the whole cell population in Group 2 than Group 1 and Group 3 (p=0.45). Mean level of p53% in the treated cases was higher compared to newly diagnosed cases (before treatment) showing a statistically significant difference (p=0.01). Higher mean levels of CD95% on whole cell population, and on CD34+ve selected cells were detected after treatment (p=0.30, p=0.83). The mean levels of p53% and CD95% were higher in BCR/ABL fusion gene positive cases than BCR/ABL fusion gene negative cases but didn't reach significant levels respectively (p=0.21, p=0.62).

Conclusion:

A- p53% and CD95% levels expression in the accelerated and blastic crisis phases of CML patients were higher than those in the chronic phase.

B- Comparative studies for the apoptotic markers with cytogenetic analysis and RT PCR techniques revealed higher levels of p53 and CD95 in BCR/ABL positive cases than BCR/ABL negative cases.

C- p53 and CD95 levels were higher in treated cases than newly diagnosed cases.

Key Words: *Chronic myeloid leukemia (CML) – p53 – CD95 (FAS) – CD34 – Apoptosis – FCM, BCR/ABL – Ph chromosome – FISH – Electron microscopy (EM).*

INTRODUCTION

Chronic Myeloid Leukemia is a clonal disease that results from an acquired genetic change in a pluripotential hemopoietic stem cell **[1]**. CML remains silent for sometime then usually transforms to a more aggressive disease after 2 to 4 years and occurs in at least two clinical

manifestations rarely accelerated phase and blastic crisis which are considered outcome changes occuring at the end of CML evolution **[2]**. Philadelphia negative cases and aberrant cytogenetic and molecular changes are usually associated with accelerated and bad prognostic sequence **[3]**.

Molecular abnormalities and mutations usually accompany the accelerated and blastic crisis phases of CML. Beck et al. **[4]**, suggested the association of abnormalities of p53 gene with acceleration of CML. Loss or mutation of the tumor suppressor gene, p53, is one of the most frequent secondary mutations in CML blastic crisis. The transition between chronic phase and blastic crisis is associated with increased resistance to apoptosis correlating with poor prognosis **[5]**.

p53 mutation is reported to be less frequent in hematologic malignancies, but when present, they are important determinants of disease outcome **[6]**. P53 has a very short half-life, so that the wild-type protein is present in the cell at undetectable levels by immunohistochemical assay. In contrast, mutated version of p53 protein tends to accumulate into the nuclei of malignant cells due to its longer half-life, thus can be detected immuno-cytochemically. Accumulation of p53 occurs sometimes without mutation, however it is also nonfunctioning **[7]**.

The level of expression of CD95 (FAS) has a bearing effect on the outcome of CML. CD95 triggering is involved in the apoptotic death of lymphoid cells and may be also important in myelopoiesis **[8]**.

Reports on ultrastructural findings showed that the study of the morphology of the blood and bone marrow cells remains one of the important criteria that the pathologists have to depend upon in the diagnosis of different hematological disorders. Besides the use of the electron microscopes by investigators in their research work, hematopathologists find it necessary in confirming the diagnosis of some hematological disorders, where other tests give inconclusive results **[9]**.

This study was conducted to explore the possible ultrastructural, molecular, cytogenetic, apoptotic and morphological abnormalities that may contribute to the progression of chronic phase of CML patients to blastic crisis phase, for focusing on high risk patients, to justify accurate lines of treatment.

SUBJECTS AND METHODS

Subjects:

This study was carried out on 30 chronic myeloid leukemia patients (18 de-novo and 12 under treatment) presenting to the Medical oncology department of the National Cancer Institute, Cairo University, in the period between 2003 and 2005 including 12 females and 18 males whose ages ranged from 20 to 60 years old with a median of 41 yrs. In addition, 10 age matched subjects were included as controls.

Methods:

All Patients were subjected to full history taking, full clinical examination, complete blood picture **[10]**, bone marrow aspiration and morphological examination **[11]**, as well as LAP score (Leucocyte Alkaline Phosphatase) **[10]**.

Specimen collection: Ten ml venous blood were collected from each patient and control, and distributed as follows: 2ml on EDTA for Flowcytometric (FCM) analysis of CD34, CD95 (FAS) and p53 **[12]** and 8ml on preservative free heparin for: Cytogenetic analysis for Ph chromosome (Ph) **[13]** and FISH **[14]** and RT PCR **[15]** and Electron microscopic examination **[16]**.

P53, CD95 and CD34 by flow cytometric analysis:

Fresh peripheral blood or bone marrow aspirate were used and analysis was performed by flowcytometer (Coulter Epics XL, Hialeh). Fluorescent labeled mouse monoclonal antibodies against human anti-Fas (CD95), CD34 and p53 fluorescein isothiocyanate (FITC) and phycoerythrin (PE) conjugated in addition to isotopic control and intrastain permeabilizing solution were purchased from Becton Dickinson (Mountain view, California).

Sample preparation:

Two ml fresh venous blood on EDTA was collected, TLC was done using coulter counter. Dilution of the sample with PBS was made to reach a total leucocytic count from 8-10x10^9/L). The test tubes were labeled as follows:

1- Control tube (isotype corresponding to each monoclonal).

2- CD34 (FITC)/CD95 (PE) tube.

3- P53 (FITC)/CD34 (PE) tube.

Detection of surface markers by direct staining:

For the CD34 (FITC)/CD95 (PE) Tube: Ten ul of monoclonal antibodies CD34 and CD95 were added to 100ul of diluted blood, and left in the dark for 20 minutes. Two ml lysis solution was added, well mixed and left for another 5 minutes. Tubes were centrifuged, cells were washed twice with PBS solution and suspended in sheath fluid to be ready for reading by flowcytometer.

Detection of intracellular markers by direct staining:

For the p53 (FITC)/CD34 (PE) Tube: Ten ul of monoclonal antibody CD34 was incubated with 100ul of diluted blood in the dark for 20 mins. Two ml of lysis solution was added and left for another 5 minutes. Cells were washed with PBS, supernatant removed and 100ul of fixative solution were added and left for 15mins, 2ml of PBS was added to cell suspension, centrifuged for 5mins, and supernatant removed. One hundred ul of permeabilizing solution and 10ul of monoclonal antibody p53 were added and left in the dark for 20mins. Cells were centrifuged for 5 minutes at 2000rpm, supernatant removed and cells washed twice with PBS and suspended in sheath solution to be ready for reading by flowcytometer. Sample was considered positive if ≥Cut off value for p53=0, cut off value for CD95 was 1.2 and CD34 was 10%.

Cytogenetic studies:

Conventional cytogenetic analysis was carried out on unstimulated bone marrow or peripheral blood cultures. Metaphases were trypsin/Giemsa-banded. Karyotypic abnormalities were described according to the specifications of the International System for Human Cytogenetic Nomenclature **[17]**. Fluorescence in situ hybridization (FISH) analysis was performed when necessary using the commercial BCR/ABL locus specific identifier (LSI) probe (Vysis Downers Grove, IL, USA) according to the manufacturer's recommendation **[14]**. For cytogenetic and FISH analysis an automated karyotyping system (Quips, Vysis, USA) was used.

Real – Time RT – PCR:

Buffy coat layer was separated by density gradient centrifugation **[18]**. Cells were preserved in RPMI 10% Demso solution to keep them viable and at -80ºC until used. RNA extract was prepared from the separated cells using pure script Total RNA Isolation kit (Gentra System, USA). BCR/ABL gene expression was carried out using Real-Time RT PCR using SYBR Green **[19]**.

The procedure began with reverse transcription of total RNA using SYBR Green RT-PCR one Step (Genecraft), with reversed primer. Primer 1: (5`-TGTGATTATAGCCTAAGA CCCGGAGCTTTT-3´). The cDNA was then used as a template for realñtime PCR with gene specific primer. Primer 2: (5`-GAGCGTGCAG AGTGGAGGGAGAACATCCGG-3´) Primer 3: (5`-TTCAGC GGCCAGTAGCATCT-GACTT-3´). The reaction mixture condition consisted of Buffer 1x, SYBR Green dye and Rox reference dye, 3.5mmol Mn-acetate solution, 0.3mmol dNTPS, 7.5pmol for each primer and 1.5uTaq polymerase DNA. The thermal cycler profile consists of: 60ºC for 60min. RT-reaction, 95ºC for 15min. DNA polymerase activation, 95ºC for 15sec. denaturation and 60ºC for 1min annealing and extension. The main PCR cycle was repeated for 40 cycles. The results were obtained in the form of PCR amplification plot curves. The Stratagene Real-Time instrument system has a built in therma lcycler and 4 laser beams directed via fiber optic cables to the wells. The fluorescence emitted travels back through the cables to a CCD camera detector.

Electron microscopic examination:

Leucocytes suspension was prepared, after separation from peripheral blood and bone marrow samples, for transmission electron microscopy according Catovsky **[20]**. The cells were fixed in 2.5% glutaraldehyde (Merk) in PBS (pH=7.4) then post fixed in 1% osmium tretraoxide (Electron microscopy Sciences) in PBS (pH=7.4) for 2 hours at 4ºC, dehydrated in ascending grades of ethanol. The cells were substituted in a mixture of Epon resin (Electron microscopy Sciences) and absolute ethanol and lastly infiltrated in three baths of Epon resin at room temperature twelve hourly. The cells were embedded in EPON 812 capsules. Polymerization of the resin then follows at 60ºC for 24 hours. Ultra thin sections were prepared and examined under the electron microscope, Joel 1200 EX II.

Statistical analysis:

Statistical Package for social sciences (SPSS) version 9 was used. Quantitative variables were

summarized using mean and SD, median, minimum and maximum values. Qualitative data were summarized using frequencies and percentage.

The relation between quantitative variables was tested by ANOVA test and Spearman Correlation. Chi or Fisher's exact tests were used whenever appropriate to test the association between the different qualitative variables. Differences were considered significant at a p value of ≤0.05 and highly significant at a p value of ≤0.01 **[21]**.

RESULTS

Thirty randomly chosen CML cases presenting to the National Cancer Institute, Cairo University, in the period between 2003-2005 and 10 age matched normal control subjects were included in this study. The age ranged from 20-60 years with a mean of 38.03±12.81 and a median of 41. Male to female ratio was 3:2 (18 males and 12 females).

Mean LAP score of all patients and controls was 32.6±57.1 (range 0 to 237). 6/30 (20%) patients had a high LAP score, they were classified as either acute blastic crisis or accelerated phase and 24 (80%) patients had a low LAP score (chronic phase of CML).

P53% by FCM:

Mean P53% level was 1.6%±2.3 (range 0 to 9.6%). Twenty four out of 30 cases (80%) showed a positive expression and 6/30 (20%) were negative for p53.

CD95% by FCM:

Mean level of CD95+ve/CD34+ve cells was 5.9%±11.7 (range 0 to 54%). Mean CD95% level studied of the whole cell population was 12.7%±23.9 (range 0 to 98%). Twenty two out of 30 cases (73.3%) expressed a high CD95% level ≥1.2 (Positive) and 8 (26.6%) expressed a low level <1.2 (Negative).

Cases were classified according to the different phases of CML into: 3 cases in acute blastic crisis, 3 cases in accelerated phase and 24 in chronic phase. Patients were divided into groups according to follow-up:

- *Group 1:* 16/30 (53.3%) chronic phase cases that remained chronic during treatment.
- *Group 2:* 12/30 (40%) chronic phase cases that developed an accelerated or blastic crisis during treatment and returned to chronic phase.
- *Group 3:* 2/30 (6.6%) chronic phase cases that developed an accelerated or blastic crisis phase then died.

Comparative studies of p53% and CD95% between the different phases of CML are illustrated in Table (1):

- There was statistically significant higher level of p53% expressed in the accelerated and/or blastic crisis phase 2.25%±2.32 and the chronic phase 2.18%±2.75 compared with that of controls 0.049%±0.04 (p=0.04).
- There was higher level of CD95% expression when measured on the whole cell population in the accelerated and/or blastic crisis 20.00%± 32.82 compared with chronic phase 15.98%± 23.84 and controls 1.27%±0.28 but did not reach significant level (p=0.14).
- On the other hand studying the level of CD95+ve/CD34+ve cells, low levels of CD95% expression were found in the accelerated and/or blastic crisis phase 10.4%±17.7 compared with the levels expressed on the whole cell population in the same phase.

Comparative studies of p53% and CD95% with BCR/ABL fusion gene expression by conventional cytogenetics, FISH and real-time RT PCR: (Table 2):

The level of p53% was higher among cases positive for BCR/ABL fusion gene 2.49%±2.64 compared with cases negative for BCR/ABL fusion gene 0.48%±0.45 but didn ot reach significant level (p=0.212).

The level of CD95% was higher among cases positive for BCR/ABL fusion gene 18.36%±27.92 compared with those negative for BCR/ABL fusion gene 10.21%±11.31, but didn't reach significant level (p=0.629).

Comparative studies of p53% and CD95% in CML groups as regards follow-up: (Table 3):

- There was no statistically significant difference in the p53% level when studied among the 3 groups of CML, though higher levels were seen among group 3 but did not reach significant level, (p=0.85).
- There were higher levels of CD95 +ve/CD34 +ve cells expressed in group 1 (9.2%±14.8) (chronic phase only) compared with group 2

(7.0%±12.2) & group 3 (4.2% ±4.2), but did not reach significant level (p=0.85).

- There were higher levels of CD95% expression when measured on the whole cell population in group 2 (26.50%±35.40) when compared with group 1 (12.32%±21.65) and group 3 (16.06%±12.53), but did not reach significant level (p=0.45).

Comparative studies of p53% and CD95% among newly diagnosed cases and their response to treatment are illustrated in Table (4):

- There was a statistically highly significant difference in the level of p53% in the treated cases (3.9%±3.0), compared to newly diagnosed cases before treatment (1.4%±1.9), (p=0.01).
- There was no statistically significant difference in the levels of CD95+ve/ CD34+ve cells in the newly diagnosed cases (before treatment) (8.4%±14.8) compared to the treated cases (7.2%±9.5), (p=0.83).
- On the other hand studying the levels of CD95% on the whole cell population, higher levels were found in the treated cases (25.6% ±28.5) compared to those newly diagnosed (before treatment) (13.8%±25.7), but did not reach statistical significance (p=0.30).

Ultrastructural findings:

EM examination of selected cases with high p53% and CD95% showed some apoptotic changes. These morphological changes were divided into early and late changes. Early morphologic changes showed chromatin condensation along the nuclear envelope with subsequent thickening and irregularity, asynchrony of nucleus with the cytoplasm, with frequent nuclear fragmentation and widening of the nuclear membranes and increase in the nuclear pores.

Late morphologic changes showed cytoplasmic changes in the form of compacting of cytoplasmic organelles, increased cytoplasmic vaculations, widened smooth endoplasmic reticulum, margination of cytoplasmic mitochondria towards the cell membrane, swelling of mitochondria and loss of its christae. as well as rufflings, blebbings and thinning of the plasma membrane (Fig. 1: D,E).

Table (1): Comparative study of p53% and CD95% mean±standard deviation between the studied groups as regards phases of CML.

	Chronic phase (a)* (n=24)	Acc. & blastic phases (a)* (n=6)	Controls (b)* (n=10)	p value
p53%	2.18±2.75	2.25±2.32***	0.049±0.04	0.049***
CD95+ve/CD34+cells	8.4±13.9	10.4±17.7	1.27±0.28	0.291
CD95%	15.98±23.84	20.00±32.82**	1.27±0.28	0.143**

*Groups with different letters are with statistical significance. ** High levels but did not reach significant level.
*** Significance <0.05.

Table (2): Comparative study of p53% and CD95% with the cytogenetics, FISH and PCR results.

	BCR/ABL Gene (-ve) (n=3)	BCR/ABL Gene (+ve) (n=20)	p value
p53%	0.48±0.45	2.49±2.64*	0.212
CD95%	10.21±11.31	18.36±27.92*	0.629

* High Levels but did not reach significant level. ** Significance <0.05.

Table (3): Comparative study of p53% and CD95% in different groups of CML as regards follow-up.

	Group 1 (n=16)	Group 2 (n=12)	Group 3 (n=2)	p value
p53%	2.00±2.71	2.35±2.48	3.05±3.46*	0.85
CD95+ve/CD34+cells	9.2±14.8*	7.0±12.2	4.2±4.2	0.85
CD95%	12.32±21.65	26.50±35.40*	16.06±12.53	0.45

* High levels but did not reach significant level. ** Significance <0.05.
Group 1: CML patients who started and remained chronic during treatment.
Group 2: CML patients who started as chronic and developed an acceleration and/or blastic crisis phase then returned to chronic during treatment.
Group 3: CML patients who started as chronic & developed an acceleration and/or blastic crisis phase then died during treatment.

Table (4): Comparative study of p53% and CD95% mean ± standard deviation as regards newly diagnosed cases and their response to treatment.

	New cases (n=18)	Treated cases (n=12)	*p* value
p53%	1.4±1.9	3.9±3.0**	0.01**
CD95+ve/CD34+cells	8.4±14.8	7.2±9.5	0.83
CD95%	13.8±25.7	25.6±28.5*	0.30

* High Levels but did not reach significant level. ** Significance <0.05.

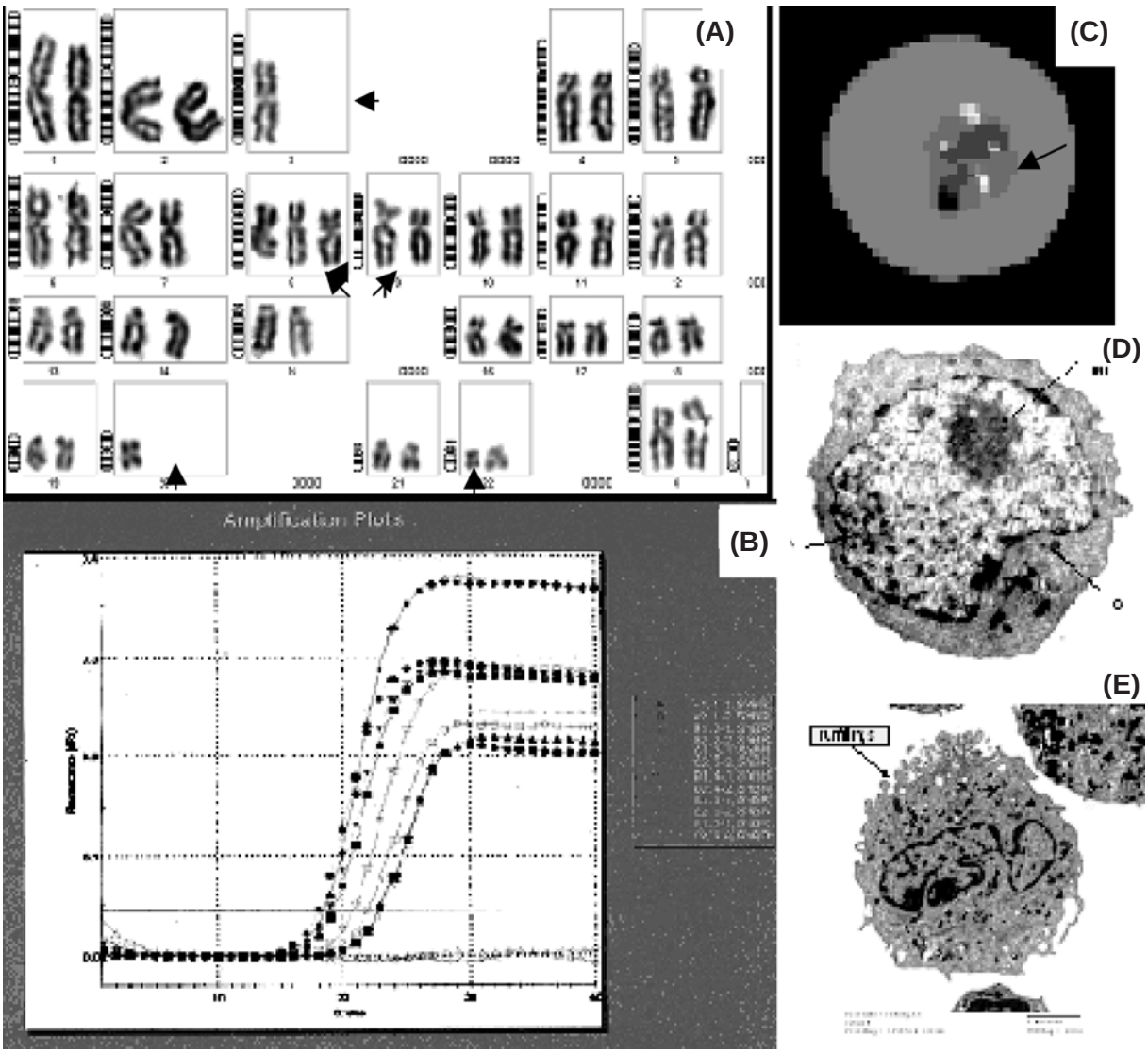

Fig. (1)

A: Karyotype of a 27 years old female presentedwith ABC on top of CML showing 45, XX, t (9;22) (q34;q11),-3 ,+8, -20.

B: PCR amplification plot.

C: FISH using BCR/ABL probe (Vysis) for a 20 years old case showing positive fusion signal.

D: An electron micrograph showing a myeloblast from a case of ABC of CML having a large immature nucleus (N) consisting mainly of euchromatin with small aggregations of heterochromatin dispersed all through the nucleus. A large prominent nucleolus (Nu) is present. The cytoplasm contains rare peroxidase positive (G) granules. The figure shows condensation of the mitochondria to one side of cell membrane (x10,000).

E: An electron micrograph of a monocyte showing increased rufflings, (x2,000).

DISCUSSION

This study was carried out on 30 randomly chosen chronic myeloid leukemia patients. Ten age and sex matched normal subjects were included as a control group.

The present study was designed to investigate the regulation of proliferation and survival of CML progenitors and the apoptotic pathway triggering in the elimination of the Ph+clone. The levels of apoptotic markers p53 and CD95 were measured by flowcytometry and compared among the different phases of CML and controls.

In this study, the mean age of the patients was 38 years and a median of 41 years, with a range of 20-60 years, most of the patients in chronic phase aged between 40-60 years and the male to female ratio was (3:2). It was found that 3 cases were younger patients of age 20–35 years presenting with blastic crisis. This finding was in agreement with previous studies **[22]**. In the present study, all the patients presented by leucocytosis with higher levels or doubling of the TLC among accelerated and/or blastic crisis phase. When compared with the control group, these findings were in agreement with previous reports **[23]**, where TLC count was $25x10^9$/L and reached $100x10^9$/L in the accelerated or blastic crisis phase.

A low LAP score was diagnostic of the chronic phase, in the present study. Only 3 cases (10%) with blastic crisis and 3 cases (10%) in accelerated phase presented with a high LAP score. This is in accordance to previous studies **[24]** which stated that the LAP score is an important diagnostic hematological data in distinguishing typical CML from other myeloproliferative disorders and reactive leucocytosis or leukemoid reactions.

p53% was expressed in high levels in the accelerated and blastic crisis phase (20% of cases) and chronic phase (80% of cases) when compared with controls with a statistically significant value (p=0.049). This is in accordance to previous studies **[25]** which reported that high percentage of p53 protein expression may indicate neoplastic transformation of chronic phase to accelerated or blastic crisis phases. Previous research **[26]** found that p53 protein expression in B-CLL is strongly associated with p53 mutation. Detection of p53 gene mutation is complex and unlike wild type p53, mutated p53 has a prolonged intracellular half-life and thus becomes detectable by immunohistochemistry and flowcytometry **[27]**. Tumour suppressor gene p53% elevations, in the accelerated and/or blastic phases, in the present study, are in agreement with a previous report **[28]** in which 14% of CML cases in accelerated and/or blastic phase expressed an elevated level of p53% whereas 65% of CML cases in chronic phase expressed low levels. These findings are in favour that alterations and elevation of p53 expression plays an important and central role in CML evolution.

In the present study, the mean level of CD95% expression on CD34+cells showed no statistically significant difference between accelerated/blastic crisis phases compared to the chronic phase and controls (p=0.29). On the other hand, high mean level of CD95% was expressed on the whole cell population in the different phases of CML but didn't reach a statistically significant difference. Higher levels of CD95% were detected among cases of accelerated, blastic crisis phases and chronic phase CML patients when compared with controls but of statistically nonsignificant value (p=0.14). These results were similar to previous reports **[29]** where the high levels of CD95% detected by flowcytometry in the chronic phase CML patients were compared to controls with statistically significant value (p<0.05), and higher levels of CD95% among accelerated phases compared to chronic phase with highly significant value (p=0.001).

In this work a comparative study was done between the p53% and CD95% expression among the studied group as regards newly diagnosed cases and their response to therapy (before and after treatment). A statistically significant higher p53% level was found among treated CML patients compared with the newly diagnosed patients (p=0.01). Higher levels of CD95% were also detected among the treated CML patients compared with newly diagnosed ones (p=0.30) not statistically significant.

p53 contributes to the cytotoxic action of many chemotherapeutic agents and protects the genome from mutagenic insult **[30]**. In quiescent cells, levels of p53 protein are low owing to its short half-life. After DNA damage, the half-life of p53 becomes prolonged **[31]** and the protein accumulates in the nucleus **[32]**, where it regulates the transcription of a number of genes.

p53 mutations typically prolong the half-life of the protein in the absence of DNA damage and are associated with increased basal levels **[33]**. However, when activated, mutant p53 protein cannot regulate gene expression because of its inability to bind to specific DNA sequences **[34]**. It was reported that IFN-alpha can greatly up-modulate Fas-R expression, an effect that seems to be more pronounced in CML cells compared to normal cells **[35]**.

The present study confirms a previous report where a high CD95% level in treated CML cases was compared with newly diagnosed cases with a high statistically significant value (p= 0.01). It was concluded that the activity of Fas triggering on hematopoietic progenitor cells derived from patients with CML is related to the levels of Fas-R induced by therapy **[36]**. The high level is possibly due to the effect of chemotherapy on apoptosis translation or RNA transcription and induction of apoptosis at all phases of the cell cycle **[37]**.

In our study the use of flow cytometry for the detection of the levels of p53% and CD95% expression, during different phases of CML patients, was an accurate method giving high statistically significant and accurate values. It was reported that flowcytometric analysis is the most precise assay because it enables the exclusion of contaminating population of non-neoplastic cells such as lymphocyte subsets and allows accurate identification of the specific immunephenotype in cases with heterogonous activity **[38]**.

Although, philadelphia negative cases and aberrant cytogenetic and molecular changes are usually associated with accelerated and bad prognostic sequence **[3]**. In this study, increased p53 expression level in BCR/ABL fusion gene positive cases compared with BCR/ABL fusion gene negative but did not reach significant levels (p=0.212). This might be due to the small number of BCR/ABL fusion gene negative cases.

In the present study, the studied group was subjected to cytogenetic analysis and real-time RT PCR techniques to define typical CML cases from atypical CML. Philadelphia chromosome (Ph) was negative in 20% of CML cases by cytogenetic analysis. Using real-time RT PCR and FISH, 10% only were negative for BCR/ABL gene confirming the results of cytogenetic analysis and the other 10% were positive for BCR/ABL.

In agreement with this comparative study, a recent study **[39]** reported that the introduction of real-time RT PCR has largely replaced the competitive quantitative procedures and more sensitive than the conventional cytogenetic method. It was stated that the fluorescence based technology enhances the reproducibility since quantitation is determined during the exponential phase of the PCR. These data were also in agreement with Xing et al. **[40]**, who described that Real-time RT PCR is a reliable more sensitive and accurate method to detect minimal residual disease, monitor the treatment outcome and predict the blastic crisis of CML patients when compared with other conventional cytogenetic and quantitative PCR techniques.

FISH technique provides an important means to complement data obtained by conventional cytogenetics. FISH has the advantage of studying larger number of cells, hence increasing the sensitivity of cytogenetic analysis **[41]**. This technique with chromosome-specific probes enables several new areas of cytogenetic investigation by allowing visual determination of the presence and normality of specific genetic sequences in single metaphase or interphase cells. In this approach, termed molecular cytogenetics, the genetic loci to be analyzed are made microscopically visible in single cells using in situ hybridization with nucleic acid probes specific to these loci **[42]**. One of the uses of FISH is in the diagnosis of CML, monitoring treatment outcome and chimerism analysis following bone marrow transplantation using XY probes if sex mismatched transplantation or BCR/ABL probes **[43]**.

Apoptosis plays a role in the pathogenesis as well as in the pathologic effects of many benign and malignant diseases. Furthermore, there is increasing evidence that the process of neoplastic transformation, progression and metastasis involves alteration in the normal apoptotic pathways **[44]**.

At the ultrastructural level, using the EM technique, the morphological features of apoptosis in blastic crisis and accelerated phase cases were studied. They were divided into early changes in the form of shrinkage of cell size,

condensation of the nuclear chromatin along the nuclear envelope, reduction of the nuclear size, nuclear fragmentation and disintegration, indenting of the nuclear membrane and nuclear splitting.

The late apoptotic changes that were detected in this study were in the form of condensation of the cytoplasmic organelles, their margination towards the cell membrane, shrunken mitochondria with loss of cristae, abnormal phagosomes in the cytoplasm, cytoplasmic vaculations, thinning and perforation of the plasma membrane and lastly the formation of apoptotic bodies which finally get released from the cell.

These findings are in agreement with the morphological features of apoptosis described by Aboul-Enein **[45]**, and Khorshed et al. **[16]**. The early apoptotic changes were described in the form of condensation of the nuclear chromatin along the nuclear envelope with subsequent thickening and irregularity, reduction in the nuclear size with frequent nuclear fragmentation. They also described the late morphologic features of apoptosis to include compacting of cytoplasmic organelles, margination of cytoplasmic organelles towards the cell membrane, rufflings, blebbings and thinning of the plasma membrane and finally nuclear fragmentation into membrane bound apoptotic bodies. These apoptotic changes could be mediated either through caspase dependent or non-caspase dependent pathways **[46]**.

In conclusion, p53 and CD95 can be used as predictors of impending accelerating and/or blastic crisis phases i.e. as tumour markers for both phases, as their expression in the accelerated and blastic crisis phases of CML patients were higher than the chronic phase. In this work, the comparative studies for the apoptotic markers in cases studied by both conventional cytogenetic analysis and PCR techniques revealed higher levels of p53 and CD95 in BCR/ABL positive cases than negative cases. Finally, we conclude that the proper diagnosis, with effective classification of CML cases by cytogenetic and molecular techniques, is essential to predict and prevent the evolution of more aggressive course among chronic phases of CML patients and to justify the accurate lines of treatment.

REFERENCES

1- Hoffbrand AV, Lewis SM, Tuddenham-Edward GD. Chronic myeloid Leukemia. Postgrad. Hematol. 1999, 4: 434-444.

2- Kantarjian H, Dixon D, Keating M, et al. Characteristics of accelerated disease in Chronic Myelogenous Leukemia. Cancer. 1988, 61: 1441-1446.

3- Lee GR, Forester J, Lukens J, Paraskevas F, Greer GP, Rogers GM. Wintrobe's Clinical Hematology. 10th ed., William and Wilkins, Baltimore Maryland, U.S.A. 1999, 23: 2508-2561.

4- Beck Z, Kiss A, Toth FD, Szabo J, Bacsi A, Balogh E, et al. Alterations of p53 and RB genes and the evolution of the accelerated phase of chronic myeloid leukemia. Leuk Lymphoma. 2000, 38 (5-6): 587-97.

5- DiBacco A, Keeshan K, McKenna SL, Cotter TG. Molecular abnormalities in chronic myeloid leukemia: Deregulation of cell growth and apoptosis. Oncologist. 2000, (5): 405-415.

6- Whight D, Oscier DG. Lypmphoid neoplasms. In: Provan D, Gribben J, (editors). Molecular Hematology. Blackwell Science Ltd., London. 2000, 99-109.

7- Natkunam Y, Lossos I, Taidi B, Zhao S. Expression of the human germinal center-associated lymphoma (HGAL) protein, a new marker of germinal center B-cell derivation. Blood. 2005, 105: 3979.

8- Munker R, Marini F, Jiang S, Savary C, Owen-Schaub L, Andreeff M. Expression of CD95 (FAS) by gene transfer does not sensitize K562 to Fas-killing. Hematol cell Ther. 1997, 39 (2): 75-78.

9- Khorshed A, Kholoussi N. Atlas of the ultra- structure of the hematopoietic cells. Tahrir Press, Cairo. 2005, 77.

10- Dacie JV, Lewis SM. Practical Haematology (Eighth edition) Churchill Livingstone London. 1995, Chpt 10 p. 176.

11- Kinney MC, Lukens JN. Classification and differentiation of leukemias. In: Wintrobe's clinical hematology, 10th ed., Williams and Wilkins, Waverly Company. 1999, 2209-2232.

12- Landy A, Muirhead K. Procedural Guidelines for performing Immunophenotyping by FCM clinical immunology. Immunopathol. 1989, 52: 4860.

13- Testa JR, Rowley JD. Chromosomes in leukemia and lymphoma with special emphasis on methodology. In the leukemic cell. 2nd ed., Churchill Livingstone, London. 1991, 184.

14- Pinkel D, Straume T, Gray JW. Cytogenetic analysis using quantitative high sensitivity fluorescence hybridization. Proc Natl Acad Sci USA. 1986, 83: 2934-2938.

15- Mc-Pherson MJ, Quirke P, Taylor GR. Polymerase chain Reaction (PCR): A practical Approach. 1st ed., IRL Press, Oxford University Press, New York. 1991, 125.

16- Khorshed A, Kholoussi N, Aboul-Enein MI, Zaki M,

Aboul Enein K. The role of scanning electron microscopy in the diagnosis of some hematological malignancies. In: Eg J Hematol. 2001, 26 (2): 26-32.

17- Mitelman F. (ed.) ISCN. Guidelines for cancer cytogenetics, supplement to an international system for human cytogenetic nomenclature. Basel: Karger. 1995.

18- Ferrante A, Thong YH. Optimal condition of simultaneous purification of mononuclear and polymorphonuclear leukocytes from human blood by the Hypaque-Ficoll method J Immunol Methods. 1980, 36: 109-117.

19- Wang X, Seed B. A PCR primer bank for quantitative gene expression analysis. Nucleic acids research. 2003, 31 (24): 1-8.

20- Catovsky D. (ed.). Ultrastructure of the Leukemic cell. In: The leukemic cell. (2nd ed) Churchill. Livingstone, New York. 1991, 427.

21- Saunders DB, Trap GR. Basic and Clinical biostatistics, 3rd edition. Connecticut, Appleton and Lang. 2001.

22- Ian R, Richard S. Chronic myeloid leukemia. Wintrobe's Clin Hematol. 2004, 2: 2235-2258.

23- Bain BJ. Chronic Myeloid Leukemias. In: Bain B.J.: Leukemia Diagnosis. Blackwell Science, London, Berlin, Tokyo. 1999, 143.

24- Enright H, Mc Glave P. Chronic myelogenous leukemia adopted from hematology basic principles and practice, Churchill Livingstone, Edinburgh, London, Melbourne and New York. 2000, 623-675.

25- Konikova E, Kusenda E, Babusikova O. Flowcytometry of p53 protein expression in some hematological malignancies. Neoplasm. 1999, 46 (6): 368-376.

26- De Angeli C, Gandini D, Cuneo A, Moretti S, Bigoni R, Roberti MG, Bardi A, Castodli GL and Del Senno L. Bcl-1 rearrangement and p53 mutation in atypical chronic lymphocytic leukemia with t (11; 14) (q13; q32) Haemotologica. 2000, 85: 913.

27- Giles FJ, Bekele BN, O'Brien S, Cortes JE, Verstovek S, Balerdi M, Yared M, Zhou X, Kantarjian HM, Keating MJ, Thall P, Albitar M. A prognostic model for survival in chronic lymphocytic leukemia based on p53 expression. British J of Hematology. 2003, 121 (40): 578.

28- Hernandez A, Hernandez P, Corral L, Muniz A, Alaezc F, Eapinosa E, Fernandez O, Martinez G. p53 gene rearrangements in chronic myelocytic leukemia. Ann Hematol. 1993, 66 (2): 81-83.

29- Carmine S, Tadatsugu S, Luigi DV, Luigia L, John AB, Bruno R, Neal SY, Jaroslaw PM. Involvement of Fas-mediated apoptosis in the inhibitor effects of interferon-a in chronic myelogenous leukemia. Blood. 1997, 89 (3): 957-964.

30- Lane DP. p53, guardian of the genome. Nature. 1992, 358: 15-16.

31- Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig R. Participation of p53 protein in the cellular response to DNA damage. Cancer Res. 1991, 51: 6301-6311.

32- Fritsche M, Haessler C, Brandner G. Induction of nuclear accumulation of the tumour suppressor protein p53 by DNA-damaging agents. Oncogene. 1993, 8: 307-318.

33- Lane DP. p53 and human cancers. Br Med Bull. 1994, 50: 582-599.

34- Prives C. How loops, sheets, and helices help us to understand p53. Cell. 1994, 78: 543-546.

35- Selleri C and Maciejewski JP. The role of Fas-mediated apoptosis chronic myeloid leukemia. Leuk Lymphoma. 2000, 37 (4): 283-297.

36- Robertson MJ, Manley TJ, Pichert G, Cameron C, Cochran K J, Levine H, Ritz J. Functional consequences of APO-1/Fas (CD95) antigen expression by normal and neoplastic hematopoietic cells. Leuk Lymphoma. 1994, 17: 51.

37- Zhang JX, Zhang Z K, Sheng H P, Tsao SW, Loh TT. Role of apoptosis in Leukemic cell lines. Life Sci. 2000, 66: 1713-1723.

38- Muller M, Lennartz K, Back B, et al. Simultaneous measurement of cellular P-glycoprotein content and function by multiparametric flowcytometry. Int J Clin Pharm Acol Ther. 2000, 38 (4): 180-186.

39- Timothy H, Susan B. Molecular monitoring of BCR-ABL as a guide to clinical management in chronic myeloid leukemia. Blood. 2005, 20 (1): 29-41.

40- Xing W, Gu BW, Zhu YM, Jiang CL, Zhoo RH, Wang AH, Sun H, Li JM, Shen ZX, Chen Z, Chen SJ. Detection of BCR/ABL transcripts in patients with chronic myeloid leukemia by real-time PCR, PCR. 2005, 85 (7): 453 -7.

41- Dierlamm J, Stefanova M, Wlodarska I, Hinz K, Maes B, Michaux L, Stul M, Verhoef G, Thomas J, De Wolf-Peeters C, Van den Berghe H, Hossfeld DK, Hagemeijer A.: Analysis of the p53, RB/D13S25, and P16 tumor suppressor genes in marginal zone B-cell lymphoma: An interphase fluorescence in situ hybridization study. Cancer Genet Cytogenet. 2000, 1; 120 (1): 1.

42- Gray JW, Kallioniemi A, Kallioniemi O, Pallavicini M, Waldman F, Pinkel D. Molecular cytogenetics: Diagnosis and prognostic assessment. Curr Opin Biotechnol. 1992, Dec; 3 (6): 623-31.

43- Frater JL, Tallman MS, Variakojis D, Druker BJ, Resta D, Riley MB, Hrisinko MA, Peterson LC. Chronic myeloid leukemia following therapy with imatinib mesylate (Gleevec). Bone marrow histopathology and correlation with genetic status. Am J Clin Pathol. 2003, Jun; 119 (6): 833-41.

44- Bold RJ, Trmuhlen PM, Mc-Conkey DJ. Apoptosis, cancer and cancer therapy. Surg Oncol. 1997, 3: 133-142.

45- Aboul-Enein MI. Principles of hematology and hemopoietic malignancy for medical under and post graduates. Tahrir press. 1998, 36-60.

46- Kolenko VM, Uzzo RG, Finke JH. Caspase dependent and independent death pathways in cancer therapy. Apoptosis. 2000, 5: 17-20.