Utility of Fluorescent *in Situ* Hybridization for Detection of Trisomy 8 in Chronic Myeloid Leukemia Patients

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ABSTRACT: *Objective*: To detect the incidence and relationship of trisomy 8 to various stages of chronic myeloid leukemia, (CML) and its clinicopathological significance**. *Patient and methods*:** Thirty five patients in different phases of the disease (15 in chronic phase, 10 in accelerated phase and 10 in blastic crisis) served as patients groups and 10 apparently healthy individuals of matched sex and age served as a control group were selected and subjected to the following; routine investigations as complete blood count, cytochemical staining, LAP score and detection of Philadelphia chromosome (Ph). Detection of trisomy 8 using fluorescent *in situ* hybridization (FISH) technique by CEP8 Spectrum Orange DNA probe AT-rich alpha satellite (centromere region) 8p11.1-q11.1 FISH was performed on all groups using either archival fixed cells, or cells from newly diagnosed cases. ***Results:*** FISH study revealed that; all patients were Ph positive, BCR-ABL fusion gene positive. Only 4 (11.4 %) out of the 35 patients with CML were trisomy 8 positive. No one (0%) in the chronic or the accelerated phase groups was trisomy 8 positive. Only 4 (40%) out of 10 patients in the blast crisis group were trisomy 8 positive. Clinically, there was statistically significant difference between patients with trisomy 8 positive and negative status as regards age of the patients. No statistically significant difference between patients with trisomy 8 positive and negative status as regards sex, peripheral blood findings and LAP-score. While there was a striking highly significant difference between patients with trisomy 8 positive and negative status as regards the progression-free survival and the overall survival time. Kaplan-Meier analysis showed poor prognosis of CML patients with trisomy 8 positive when compared to trisomy 8 negative patients. ***Conclusion:*** Detection of trisomy 8 by FISH technique correlates with the duration of remission and overall survival rate in CML patients. It is useful as a supplement to standard cytogenetic studies to identify high risk patients and can be incorporated into management decisions.

1. Introduction

Chronic myeloid leukemia is a clonal malignant disorder arising in a transformed hematopoietic stem cell (HSC). Chronic myeloid leukemia is caused by the translocation of chromosomes 9 and 22 to create the Philadelphia chromosome (Ph). This translocation removes a critical regulatory domain from the tyrosine kinase, this means that the cell escapes the constraints of normal cell growth and proliferates uncontrollably. Disease progression and blast crisis CML is associated with characteristic cytogenetic and molecular events [1]. The secondary chromosomal aberrations that occur as part of clonal evolution are demonstrable in 60% to 80% of cases of CML in accelerated and blast crisis phases. The secondary changes in blast crisis phase usually are complex, with trisomy 8, an extra Ph and isochromosome 17q most common, occurring in 34%, 30%, and 20% of cases, respectively [2].

Trisomy 8 is one of the major anomalies additional to the t(9;22), with i(17q), + der(22), +19. These additional anomalies may be present at the diagnosis of CML (in 10%, possibly with unfavorable significance), or may appear during course of the disease, they do not

indicate the imminence of a blast crisis, although they also frequently emerge at the time of acute transformation. Trisomy 8 is more often found in the myeloid than in the lymphoid blast crisis [3].

Trisomy 8 is also common in blast crisis (approximately 40%), since c-MYC is located at 8q24, it is tempting to speculate that c-MYC is driving progression. There are several lines of evidence linking c-MYC to progression. In vitro inhibition of c-MYC with antisense oligonucleotides, or dominant-negative constructs, can inhibit BCR-ABL transformation or leukemogenesis. c-MYC is often overexpressed in blast crisis compared with chronic phase [4]. While in patients with accelerated myeloid leukemia (AML) with trisomy 8, c- MYC is down-regulated, but other genes on chromosome 8 are upregulated [3]. Curiously, trisomy 8 is a common feature of cases of clonal evolution in patients with CML treated with imatinib who are in cytogenetic remission. These cases with trisomy 8 seem to have a benign course, suggesting that trisomy 8 in and of itself may not be leukemogenic [5]. Emergence of trisomy 8 in Ph negative cells during the course of

imatinib treatment is transient and not related to therapy-related myelodysplasia or acute leukemia [6].

The aim of this work is to detect the incidence of this chromosomal abnormality (trisomy 8) in patients with CML and its clinicopathological significance.

2. Patient and Methods

This was a prospective study done between August 2005 to September 2008 at Clinical Pathology Departments, Tanta and Banha University Hospitals. The patients had treated and followed up at Clinical Oncology Department, Tanta University Hospital.

This study included 35 Ph positive patients diagnosed as CML and 10 healthy individuals of matched age and sex (Table 1). All patients were classified into 4 groups; group I included 15 patients in chronic phase (8 males & 7 females), group II included 10 patients (8 males & 2 females) in accelerated phase and group III included 10 patients (6 males & 4 females) in blast crisis phase and group IV included 10 healthy individuals (4 males & 6 females) as a control group.

Table (1): Age distribution among studied groups:

Age groups	Range (years)	Mean ± SD	ANOVA test	P–value
Group I	21 - 53	41 ± 11		
Group II	20 – 53	35 ± 11	2.75	> 0.05
Group III	35 - 76	51 ± 16		
Group IV	23 - 64	43 ± 12		

Diagnosis of patients in chronic phase of CML is based on clinical evaluation and laboratory assessment in the form of leucocytosis more than $20,000/cm^3$, absolute basophilia, eosinophilia, thrombocytosis, elevated serum lactate dehydrogenase (LDH), decrease neutrophil alkaline phosphatase (LAP score), bone marrow aspiration shows granulocytic hyperplasia, basophilia, megakaryocytic hyperplasia, and the Ph chromosome positive.

Diagnosis of accelerated phase was based on laboratory assessment: leucocytosis, peripheral basophilia, persistent thrombocytopenia, persistent thrombocytosis, and 10-19% blasts of peripheral white blood cells or bone marrow cells unresponsive to therapy.

The blast crisis phase was diagnosed by more than 20% blast of peripheral blood or marrow and/or extramedullary blast proliferation [7].

The exclusion criteria; were patients with hereditary blood disease, chromosomal anomalies other than Ph chromosome and with congenital anomalies. All the studied groups were subjected to the following: Full history taking, proper clinical examination, complete blood count, kidney function (blood urea, serum creatinine, uric acid) and liver function (total bilirubin -AST- alkaline phosphatase) testes.

Cytochemical staining, detection of Ph chromosome and numerical abnormalities in chromosome 8 using FISH technique by CEP8 Spectrum Orange DNA probe AT-rich alpha satellite (centromere region) 8p11.1-q11.1 [Vysis Inc. (Abbott Molecular Technical Services Department, USA)] were carried out.

FISH was performed on all the patients using either archival fixed cells, or cells from newly diagnosed cases.

Fluorescent in situ hybridization; the target DNA in the chromosome to be analyzed is denatured and hybridized to the chemically modified (or heat denatured) single stranded nucleic acid (probe) sequence (fluorophore labeled) that are complementary to the genomic DNA sequences to be targeted. The reaction conditions are adjusted so that hybridization only occurs between probe and target DNA sequences of high complementarity. Target DNA is made visible by counter staining with a DNA-specific fluorescent dye that emits in a different special spectral range [8].

Procedure; Cell culture: separation of chromosomes from cells of peripheral blood samples depends on allowing cells to proliferate in culture media followed by treatment with a mitotic arrest agent as colcemid which obstruct the formation of spindle fibers and arrest cell proliferation at the metaphase. Then the cells must be treated with hypotonic (KCl, 0.56%) solution for swelling of the cells and adequate spreading of the chromosomes, the cells must be fixed in this state using fresh fixative solution and stored till used for karyotyping or FISH technique [4].

Rapid cell culture; under laminar follow, for each patients and controls, 1ml of Na-heparinised peripheral blood (Na. heparin) added to 5ml culture media RPMI 1640 supplemented by L-glutamate, fetal bovine serum 20%, penicillin-streptomycin, with phytohemagglutinin (PHA) was cultured in sterile plastic tube and incubated for 24 hours.

Harvesting and fixation; colecemid was added to each tube, incubated at 37°C for 45 minutes. Then the tubes were mixed centrifuged and most of the supernatant was discarded leaving only 0.5 ml to resuspend the cell pellet. Hypotonic solution (KCl, 0.56%) was added to each tube, incubated at 37°C for 20 – 30 minutes, then centrifuged and supernatant was discarded. Few drops of freshly prepared cold fixative were added to the suspension, then centrifuged and washed repeatedly until supernatant became clear. The cell pellet was suspended in the fixative and stored at -20 °C until used for FISH analysis.

Slide preparation; one to two drops of fixed cell suspension were added on a clean slide then left to dry for 10 minutes. Slides viewed under a phase contrast microscope, the number of interphases per field was examined under low power. A minimum of 100 cells

pre field is required for optimum assay results. Cells should be well fixed, well spread and should not be clumped to ensure efficient hybridization and easy interpretation.

(1) Preparation and application of the probe; the probe was mixed by vortex and added to the marked area on the slide. Then the slide was covered immediately with cover slip and sealed with rubber cement.

(2) Denaturation of specimen DNA and the probe (codenaturation); denaturation of the probe and specimen occur at the same time. The specimen slide was placed on the HYBrite instrument (HYB) melting temperature 74°C for 2 minutes for denaturation then hybridization overnight at 42°C.

(3) Post hybridization wash; the slides were washed first in 0.4× SSC (standard saline citrate)/0.3NP-40 at room temperature. In each wash, the slides were incubated for 2 minutes, then removed and air dried in dark.

(4) Counterstaining and signal detection; counter stain DAPI II (diamidine phenyl indol dihydrochloride II) were added at the marked area of the slides, then clean cover slips were applied and sealed well. Then the slides were placed in a dark box in a fridge for 20-30 minutes before screening and viewed under the fluorescent microscope using oil immersion objective.

FISH analysis were used on interphase nuclei and at least 200 interphase nuclei were scored. Normally chromosome 8 centromeric probe by FISH on interhpase nuclei reveals two distinct orange signals. While in cells possessing trisomy 8 three distinct signals were seen.

FISH study were carried out for control group, 500 non-overlapping nuclei with intact morphology were scored to determine the number of hybridization signals for trisomy 8. Then the mean value and standard deviation of the signals / 100 cells was calculated and based on mean+2 SD, our calculated cut off value was 4.6%. So if the percent of cells showed trisomy 8 signals were > 4.6% the case considered as positive but if the signals < 4.6% the case considered as negative.

Protocol of treatment; treatment regimens for the patients with CML depend on the phase of their disease (chronic, accelerated, or blast crisis phases)

Chronic phase: Initial treatment therapy used for the studied patients presented with chronic phase was; hydroyurea (hydrea) 40mg/kg/day ; this was reduced by 50% when the WBC count drops below 20×10^9/L. Busulfan (myleran) 0.1 mg/kg/day until WBC count decrease by 50%, at which point the dose is reduced by 50%. Therapy was dicontinued when the WBC count drops below 20×10^9/L and was restarted when the count

rises above 50×10^9/L. Interferon-alfa (IFN-a) with a dose of 5 mIU/m^2 daily subcutaneously.

Accelerated and blast crisis phases: For patients presented with accelerated or blast crises phases the initial therapy was; cytarabine (Ara-C) 10 mg/m^2 /day intavenous injection over 10 days per month ,intensive chemotherapy regimens including high dose Ara-C and doxorubicin or daunorubcin ,interferon-alfa (IFN-a) with a dose of 5 mIU/m^2 daily subcutaneously. Tyrosine kinase inhibitor imatinib (Gleevec) with a starting dose of 400 mg per day had received for only two patients as this standard drug for treatment of CML was not available to the rest of our patients at the time of the study. None of the studied patients had undergone bone marrow transplantation.

Statistical methods; data was statistically analyzed using SPSS (Statistical Package for Social Science) program version 13 for windows and Epi info program. For all the analysis p value <0.05 was considered statistically significant [9]. The statistical methods used were Chi square test, Fischer exact test, Student t-test and ANOVA test.

Survival analysis done using Kaplan meier curve to detect the prognostic significance of the studied groups [10]. Progression free survival was measured from the date of diagnosis of the patients to the date of progression to acceleration or blast crisis phase**s** and overall survival from the date of diagnosis to the date of last follow up or the date of last seen alive**.**

3. Results

This study revealed that; CML patients showed anemia, leucocytosis, increasing basophils and blast cells percentage with progression to acceleration and blastic crisis. In all patients LAP-score was decreased.

No statistically significant difference between patients with trisomy 8 positive and negative as regards sex (Table 2). While there was statistically positive difference between the age of trisomy 8 positive and negative patients (Table 3). There was a statistically significant difference between the mean value of hemoglobin (Hb) level in negative trisomy 8 cases (9.1±2.08) and positive cases (6.7±0.9) where *p*<0.05. Also a statistically significant difference of total leukocytic count (TLC) between trisomy 8 positive and negative cases (*p*<0.05) (Table 4).

Table (2): Comparison between sex and trisomy 8 status in the studied groups:

Sex	Trisomy 8		*p*-value	F-test
	-ve No (%)	+ *ve* No (%)		
Male	2 (50.0)	18 (58.1)	> 0.05	0.05
Female	2 (50.0)	13 (41.9)		
Total	4 (100)	31 (100)		

Table (3): Comparison between Age and trisomy 8 status in the studied groups

Trisomy 8 status	No	Age range (years)	Mean ± SD	T-test	p–value
Negative	31	20 – 64	40 ± 11	3.5	< 0.001*
Positive	4	35 - 76	62 ± 18		

* Significant

On the other hand, there was no statistically significant difference in platelet (PLT) count between trisomy 8 positive and negative cases ($p>0.05$). Also, there was no significant difference between the percent of blast cells in peripheral blood (PB) and trisomy 8 status among the studied groups (Table 4).

No patients (0%) in group I (0/15) or group II (0/10) were positive for trisomy 8. While 40% (4/10) of patients in group III (blast crisis group) were trisomy 8 positive. The later represent 11.4 % (4/35) of all CML patients in our study (Table 5). All patients were Ph positive. Distribution of BCR-ABL fusion gene percentage in 200 examined cells of studied groups in which all patients were BCR–ABL positive with mean value of (94.6±2.9) in group I, (92.8±2.3) in group II, and (94.1±3.8) in group III. The difference was insignificant as $p>0.05$.

There was a striking highly significant difference between patients with trisomy 8 positive and negative as regards the progression-free survival (Table 6) it ranges from 48 to 70 months with mean value of (61±5.5) for trisomy 8 negative group and from 24 to 40 months with mean value (31±7) for trisomy 8 positive group ($p<0.001$). As regard the overall survival, the results of this study revealed a statistical significant difference between overall survival in months and trisomy 8 status in which it ranged from 47 to 84 months (67±9) in trisomy 8 negative group and from 33 to 44 months (37±5) in trisomy 8 positive group. The difference was significant where $p<0.001$ (Table 7).

Kaplan-Meier analysis showed poor prognosis of CML patients with trisomy 8 positive when compared to trisomy 8 negative patients as regard the progression free survival and overall survival rates (figs. 2 & 3, respectively).

Table (4): Comparison between TLC, PLT×10³⁄mm³ , Hb (gm%) and blast cells (%) with trisomy 8 status in studied groups

Parameter	Trisomy 8	Range	Mean ± SD	ANOVA	
				T	p-value
TLC× 10^3⁄mm³	Negative +8	5 – 427	177.7 ± 124.7	3.2	<0.001*
	Positive +8	276.6 - 500	384.15 ± 91.5		
PLT× 10^3⁄mm³	Negative +8	50 – 786	276.9 ± 230.1	0.3	>0.05
	Positive+8	55 - 623	92 ± 5.5		
Hb	Negative +8	5.9-13.8	9.1 ± 2.08 gm%	2.2	< 0.05*
	Positive +8	5.6 –7.7	6.7 ± 0.9 gm%		
Blast cells	Negative +8	2 – 40 %	15 ± 12 %	1.6	> 0.05
	Positive +8	21 – 30 %	24.5 ± 4 %		

TLC; Total leucocytic count, PLT; Platelets cont, Hb; Hemoglobin.

Table (5): Distribution of trisomy 8 among the studied groups

Trisomy 8	Groups			X² test	p-value
	Group I No (%)	Group II No (%)	Group III No (%)		
Positive	0 (0.0)	0 (0.0)	4 (40.0)	11.3	<0.001*
Negative	15 (100)	10 (100)	6 (60.0)		
Total	15 (100)	10 (100)	10 (100)		

* Significant

Table (6): Comparison between progression free survival rate with trisomy 8 status in studied groups

Progression free survival	Range (month)	Mean ± SD	t-test	p–value
Trisomy 8				
Negative	48 – 70	61 ± 5.5	9.9	< 0.001
Positive	24 - 40	31 ± 7		

Table (7): Comparison between the overall survival rate in months and trisomy 8 status in studied groups

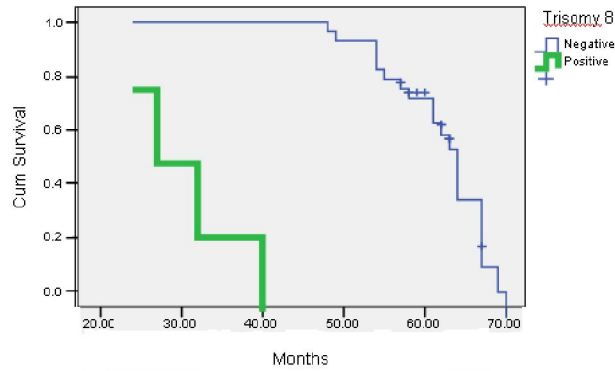| Overall survival	Range (month)	Mean ± SD	t-test	p–value
Trisomy 8				
Negative	47 – 84	67 ± 9	6.7	< 0.001*
Positive	33 - 44	37 ± 5		

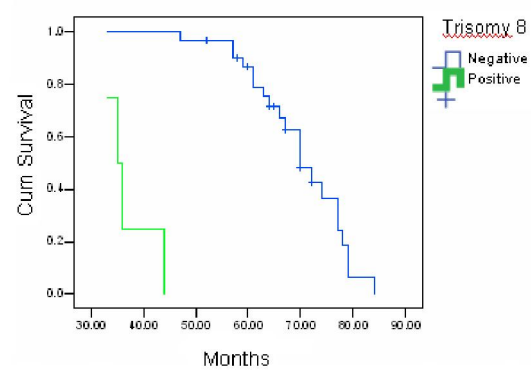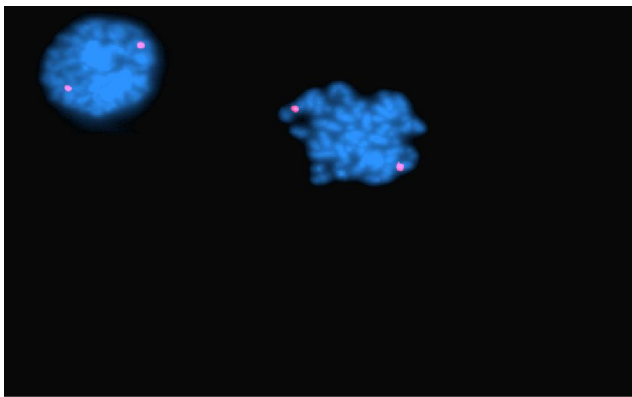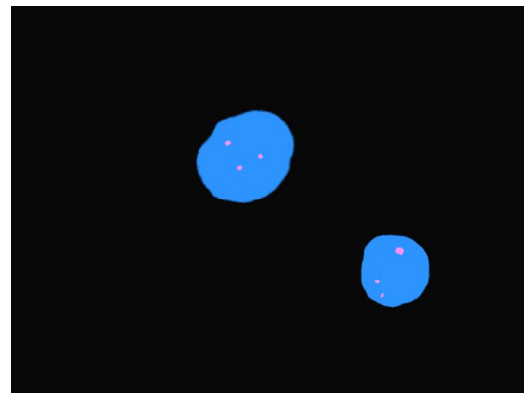**Figure (2): Kaplan-Meier analysis of the progression-free survival**

Figure (3): Kaplan- Meier analysis of the overall survival

Picture (1): A case of CML showing one metaphase cell and one interphase cell with normal chromosome 8 (two signals)

Picture (2): A case of CML with trisomy 8 positive cells (three signals).

4. Discussion

In the current study trisomy 8 was detected in 11.4% (4/35) of all CML patients. The four positive cases were found in group III (blast crisis) and they represent 40% (4/10) of the cases in this group. Similar results were reported by Radich *et al.* [11]. In other study by Mitelman *et al.*,[2] trisomy 8 as secondary changes in CML was occurring in 34% of blast crisis phase. Moreover, in Zimonjie *et al.* [12], the percentage of trisomy 8 as secondary genetic abnormalities in blastic phase of CML was 38%. The same percentage was found by Calabretta and Perrotti,[13].

Also, trisomy 8 was detected as secondary genetic abnormality in blastic phase of CML by Johansson et al.,[14] who reported that in most instances, the t(9;22) is the sole chromosomal anomaly during the chronic phase of the disease, whereas additional genetic changes are demonstrable in 60-80% of cases in blast crisis phase. The secondary chromosomal aberrations are clearly nonrandom, with the most common chromosomal abnormalities being trisomy 8 positive (34%) of cases with additional changes.

Since c-MYC is located at 8q24, it is tempting to speculate that here c-MYC is driving progression. There are several lines of evidence linking c-MYC to progression. Indeed, c-MYC is occasionally amplified and over-expressed in CML-BC, but there is no clear-cut correlation between trisomy of chromosome 8 and c-MYC amplification/over-expression. Nevertheless, treatment of CML-BC cells with c-MYC antisense oligodeoxynucleotides suppressed in vitro colony formation and in vivo leukemogenesis, consistent with a role of c-MYC over expression during disease progression. [15]. This suggestion is also supported by the results of the study done by Raida *et al.* [16] who reported that hybridization spots for the c-MYC locus were consistent with the chromosome 8 interphase FISH results in each of the Ph positive CML patients tested in this study.

On comparing the BCR−ABL fusion gene percentage with the trisomy 8 status, we didn't find a statistically significant difference. This agrees with the results of other studies done by Huntly *et al.* [17], Lee *et al.* [18] and Costa *et al.* [19].

The present work revealed that there were no statistically significant differences between trisomy 8 positive and negative groups as regard, sex, WBCs count, platelets count, Hb% and LAP-score. These results are similar to the results reported by Sinclair *et al.*, [20] and Huntly *et al.* [21]. As regard the age of the patients, there was a statistically significant difference between trisomy 8 positive and negative groups where the mean value of age in positive cases (62±18 years), and in negative cases (40±11). Such finding could be either from the increasing instability associated with aging, or with accumulated genotoxic insults that occurs over time and thus coincident with chronologic aging.[4]

The survival data of patients with and without trisomy 8 were analyzed by Kaplan-Miere survival analysis. A striking statistical significance was found. The estimated median survival time for patients with trisomy 8 positive was (31 months) while it was (61 months) in trisomy 8 negative patients. A marked statistical significance difference was detected in the progression free survival, between the two groups. The mean progression free survival time was 31±7 months in trisomy 8 positive cases and 61±5.5 months in trisomy 8 negative cases. These results were in agreement with the study done by Elliott *et al.* [22].

Similarly, when we compared the overall survival in trisomy 8 positive and negative groups there were a statistically significant difference between them. The mean overall survival time was 37±5 months in trisomy 8 positive patients and 67±9 months in trisomy 8 negative patients. This was in agreement with De Botton *et al.*, [23] in which patients with trisomy 8 positive as the sole aberration, the median survival time was 12.5 months. According to Huret [24] the median survival time ranged between 13 and 20 months. However, individual studies showed a wide variation in the results.

Survival was significantly poorer when a high proportion of the mitotic cells were trisomic[25]. Jennings *et al.* [26] suggested that increases in c-MYC were important to the course of the disease and that trisomy of chromosome 8 was an alternative means for achieving amplification of this gene. Another explanation was attempted by Virtaneva *et al.* [3] who suggested that greater resistance to apoptosis might account for the reported resistance of acute myelogenous leukemia (AML) trisomy 8 positive patients to cytarabine chemotherapy

5. Recommendations

An interphase FISH studies for BCR-ABL gene should be performed in conjunction with classic cytogenetic studies in the diagnostic evaluation of CML patients to identify high risk patients and should be incorporated into management decisions.

An interphase FISH studies for numerical chromosomal abnormalities such as trisomy 8 should be performed to detect secondry cytogenetic evolution that occurred as the CML progress from chronic to blast crisis phases.

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