Cytogenetic Response of Peripheral Blood in Chronic Myeloid Leukemia Patients Treated With Imatinb

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ABSTRACT:

BACK GROUND:

Chronic myeloid leukemia (CML) is a stem cell disorder associated with an acquired chromosomal abnormality, Philadelphia chromosome (Ph), which arises from the reciprocal translocation of part of long arm of chromosome 9, in which proto-oncogene ABL gene (ablson) is located, to long arm of chromosome 22, in which BCR gene (break point cluster region) is located forming BCR-ABL fusion gene. The suppression of BCR-ABL is likely to be crucial for therapeutic success. The development of the BCR-ABL-targeted Imatinib mesylate represents a paradigm shift in the treatment of CML.

OBJECTIVE:

This is a prospective study designed as a try to apply cytogenetic technique as a conformational diagnosis of Philadelphia chromosome (Ph) in CML patients and also, to follow up CML patients treated with imatinib mesylate (IM) for assessment of cytogenetic response of peripheral blood at different IM treatment duration.

METHODS:

Prephral blood samples were collected from CML patients every 3-6ms. At first, (310) prephral blood(PB) samples related to 135 CML patients were cultured but only 181(58 %) cultures related to (42) patients were successful (gave obvious metaphases). The degree of cytogenetic response of peripheral blood was quantified according to the proportion of Philadelphia chromosome positive metaphases.

RESULTS:

The results showed that (64.28%) of CML achieved major peripheral blood cytogenetic response while (35.71%) achieved partial cytogenetic response.

CONCLUSION:

Conventional cytogenetic karyotyping is necessary for Ph–chromosome detection and also, as an assay for periodical assessment of cytogenetic response in CML patients treated with imatinib. Imatinib has resulted in cytogenetic responses in first line IM treated patients and in those who have failed previous IFN-α therapy and in CML patients at early and late chronic phase.

KEYWORDS: chronic myeloid leukemia-philadelphia chromosome-imatinib-cytogenetic response.

INTRODUCTION:

A reciprocal translocation occuring between chromosome 9 and chromosome 22 result in the typical t(9; 22) giving rise to Ph-chromosome and it is found in about 95% of cases of CML. A minority of cases has a simple variant translocation (involving either chromosome 9 or chromosome 22 but not both) or a complex variant translocation (with the involvement of chromosomes 9, 22 and a third chromosome) [(1).] About, 60-80% of patients with CML develop additional non random cytogenetic abnormalities often several months before the development of advanced phases such blast crisis, the commonest abnormalities are trisomy of chromosome 8(+8), isochromosome i(17q), trisomy of chromosome (+19) and double Ph-chromosome (+ph) followed by deletion in chromosome 7(–7),(–17)and t(3;21). Based on the frequency of the combinations in all metaphases and subclones, it has been suggested that i(17q) and trisomy of chromosome 8 are early changes, whereas trisomy of chromosome 19 might occur late during disease progression [(2,3).]

Secondary abnormalities are common in myeloid transformation than lymphoid transformation. The abnormalities most often associated with lymphoid blast crisis are (-7) and t (7) (q22). Abnormalities most often associated with myeloid transformation are i(17q), +8 ,t(3;21) (q26;q22), inv(3)q21q26 and (-13)(q12q14) [(1, 3)] . At the beginning of the 1980s,

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interferon- α , the Immune modulator and a hypoproliferative agent,was introduced as a therapy for CML and produced sustained cytogenetic responses in up to one-third of patients [4].The initial single center results were subsequently confirmed in randomized trials that demonstrated a survival advantage for interferon- over hydroxyurea and busulfan [5,6]. A large randomized trial suggested that the combination of interferon- and cytarabine is superior to interferon-α alone [7], a finding that was not confirmed in a subsequent study [8]. The cytogenetic remissions induced by interferon-α are durable in a proportion of patients, sometimes even after discontinuation of the agent [9]. But with the use of a very sensitive molecular assays, BCR-ABL mRNA is still detectable, these long-lasting remissions amount to a biological although not molecular cure of the disease [10]. The essential role of BCR-ABL tyrosine kinase activity for cellular transformation provided the rational for targeting this function particularly [11].By late of 1980s, imatinib, a synthetic competitive inhibitor of ATP binding with some degree of specificity to tyrosine kinase had been generated. Many studies demonstrated that imatinib inhibits all ABL kinase at submicromolar concentration including $p210^{BCR-ABL}$, $p190^{BCR-ABL}$, v-ABL and c-ABL tyrosine kinases. So, treatment with imatinib decreases the number of colonies of CML cells formed and increasing the growth of BCR-ABL negative progenitor cell. Imatinib appears to be selectively toxic to cells expressing the constitutively active BCR-ABL protein tyrosine kinase and not normal progenitors and subsequent experiments showed that continued exposure to imatinib for CML patients is necessary to eradicate the tumor or to maintain the complete molecular response in the absence of resistant mutations [12].

PATIENTS AND METHODS:

Patients This is a prospective study enrolled 135 CML patients at The National Center of Hematology (NCH) /Al-Mustensseria University from February 2006 to August 2008. They were diagnosed clinically and hematologically (in the hematology lab at the center mentioned above) as CML. Also, healthy individuals [15] were included as CML negative control. Patients were randomly selected concerning to age, gender, disease duration, disease phase and pre-treatment.

Sample collection Three ml of peripheral blood (PB) samples were obtained from 310 CML patients and 15 healthy individuals and were placed in vacutenar tube containing heparin (final concentration 50U/ml).

Methods Blood culturing Peripheral blood was cultured as in method described by Czepulkowski [13]. About 0.5ml of heparinized PB was added to 5ml of sterile complete RPMI 1640 cultured media; contain Phytohemeagglutinin (sigma, USA) and 20% fetal calf serum (Impoil, USA). Cultured tubes were mixed gradually by inversion.The information such as patient's name; time and date of culturing were mentioned on each tube. Cultured tubes were incubated at 37°C for 72 hr using Isotemp. Incubator (Fisher Scientific, Germany) with inverting each 24 hr. Then, 0.1ml of colcimied was added to give a final concentration of 0.02µg/ml and incubated at 37°C for 30 min. After that, cultured cells were harvested.

Harvesting of blood cultures Following colcimied treatment, the cultured cells were centrifuged at 1500rpm for 10 min, using bench top Centrifuge (Jouan, C 4i, France). The supernatant was removed and the cell pellet was re-suspended by adding 10ml of pre-warmed hypotonic solution of KCl (0.075M) gradually with tapping, then incubated at 37°C for 25 min.Cultured cells were centrifuged. Few drops of chilled fixative solution (absolute methanol (Analar, UK): glacial acetic acid (Analar,UK),3;1 v/v) were carefully added with constant agitation to avoid clumps forming. Further fixative solution was added to volume of 10ml and then, the tubes were incubated at –20°C for at least 30 min. Then, cell suspension was centrifuged. This step was repeated at least for 3 times, but incubation temperature and time after adding fixative solution was at 25°C for 10 min.Cells suspension was used in slide preparation or stored at –20°C until used.

Slide preparation Cells suspension that has been stored at –20°C was centrifuged, the supernatant was removed and replenished with fresh fixative solution, Cultured tubes were re-centrifuged, the supernatant was removed just above the cell pellet and few drops of fresh fixative solution was added, approximately (0.5-1)ml, depend on the pellet size. Clean wet slide was held at corner position 45° with forceps and the cells suspension was dropped vertically from nearly 100cm above the slide.

At least, 2-5 slides were prepared for each sample. Slides were labeled and left to dry at 25°C then stained. **Giemsa staining** Giemsa stain stock solution was prepared as described in Franke and Oliver[14].Giemsa stain working solution was prepared by mixing 1ml of Giemsa staining stock

solution, 1.25 ml of absolute methanol, 0.5 ml of 0.5M Sodium bicarbonate and 40 ml of dH2O .

A-Non- Banding method Prepared slide was held horizontally and enough quantity of Giemsa stain working solution was added to cover all the surface of slid for 5min. Slide was washed with pre-warmed PBS and hold vertically to dry.

DPX mountain was added and cover slip used to cover the slid. Then, slide was examined using light microscope (Olympus, Germany) at 40X magnification and then 100X magnification.

B- Banding method Prepared Slide was dried in an oven (nüve FN400, Turkia) at 68°C for 10 min, held horizontally and enough quantity of 0.125% cold trypsin solution was added to cover all the surface of slide at 25°C for 15-20sec.

Then, Slide was washed with pre-cold Sornson`s buffer and the buffer was removed by tapping the slide. Slide was hold horizontally and stained with Giemsa stain working solution for 15-20 sec and washed with pre-warmed PBS. Slide was hold vertically to dry at 25°C and mounted.

RESULTS:

Conventional cytogenetic study was performed using standard technique for culturing PB of CML and healthy individuals, table (1).

Cytogenetic analysis was done every (3-6) months for CML patients during the follow up for detecting Ph-chromosome and assessment of cytogenetic response of peripheral blood to IM treatment. At least 20 metaphases were analyzed for detecting Ph-chromosome. Successful culture referred to that culture obtained obvious metaphases. The degree of cytogenetic responsiveness of peripheral blood was qualified according to the proportion of Ph–positive metaphases, as complete cytogenetic response (CCyR) (0 %), major cytogenetic response (MCyR) (1-35) %, partial cytogenetic response (PCyR)(36-65)% and no cytogenetic response (NCyR) (≥ 66%). MCyR and PCyR were achieved in (27/42)(64.28%) and (15/42)(35.71%) of CML patients, respectively, after different IM treatment durations.The relationship between the degrees of CyR with the treatment was represented in table (2). Most of patients who had achieved MCyR and PCyR were newly diagnosed. All the patients who achieved MCyR were in CP, and were in CP from diagnosis until the end of this study. Nearly, two third of patients who achieved PCyR were previously treated with other treatments. Of them, 8 patients were in CP and 7 patients were in AP. figure (1) shows Ph-positive CML patient. The relationship between the percentage of CML patients who achieve MCyR or PCyR and IM treatment durations was shown in figure (2). As seen, the peak value of patient's percentage who achieved MCyR was during 24 ms. There was a significant differences between the percentage of patients who achieved MCyR or PCyR through different IM treatment duration (X2=9.31, p =0.01) and (X^2 =11.60, p= 0.01), respectively. The mean daily IM doses that was administrated to patients who achieved MCyR was 300 mg, while the mean daily IM doses that were administrated to patients who achieved PCyR was (357.14±14.69) mg. Before the end of this study by eight months, IM daily dose was escalated for all patients to at least 400 mg. The mean gap duration in IM treatment for patients who achieved PCyR was nearly double that of patients in MCyR (p=0.05, LSD 8.29).

There was no significant differences between those two groups in the mean gap duration in IM treatment with alternative treatment (LSD =3.56) but the duration of gap in IM treatment without any alternative treatment for patients who achieved PCyR was three times more than those in MCyR (p=0.05, LSD=2.92).

Table 1: Cytogenetic analysis of prephral blood samples

Periphral blood sample from	No. of patient	No.of sample collected	No.of cultured samples	No.of success cultured samples / No.of patients	Percentage of succful cultured samples (%)
CML	135	310	310	181/42	58.38
Healthy individuals	15	15	15	8/8	53.33

- CML=Chronic myeloid leukemia

Table 2: Pre-treatment and cytogenetic response

First line IM	MCyR n (%)	PCyR n (%)	X^2-value
	10(71.43)	4(28.57)	8.15**
previously treated	17(60.71)	11(39.29)	6.83*
X^2-value	3.13ns	3.06ns	----

- * = ($p < 0.05$) , **= ($p < 0.01$), ns = non-significant, MCyR = major cytogenetic response, PCyR = partial cytogenetic response, IM = Imatinib, X^2 = Chi–square value.

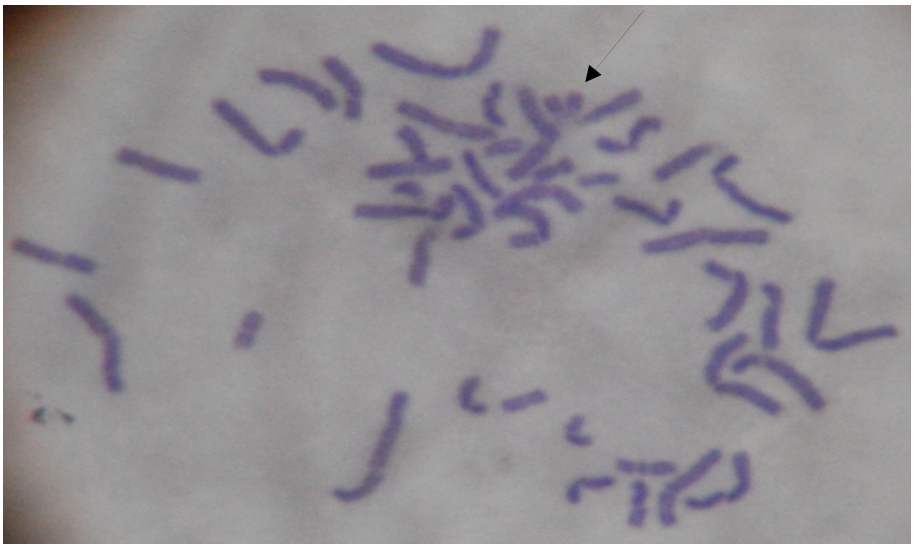

Figure 1: karyotype of PB metaphase of CML patient.The arrow indecated Ph-chromosome. Chromosomes were stained using Gimssa stain and visualized under 100X magnification power.

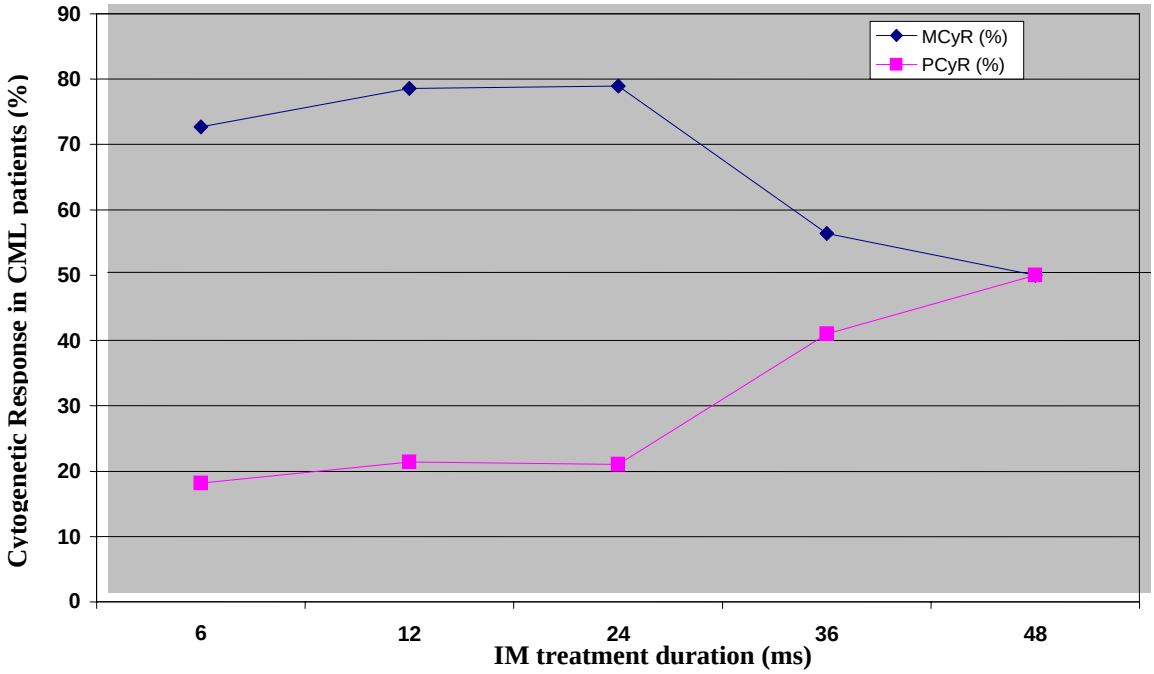

Figure 2: Cytogenetic response during different IM treatment duration.

DISCUSSION:

Cytogenetic response to Imatinib

Monitoring the percentage of Ph positive cells is the best validated system for the assessment of the response to tyrosine kinase inhibitors, since the cytogenetic response is the best surrogate marker of survival [15, 16]. For patients who achieve a CCyR to IM, the five years survival rate is close to 100% [17, 18]. Only (58.38%) of blood cultures from CML patients were successful. Other cultures failed some of them repeatedly, even when the sample was collected within different intervals. That may be due to hypocellularity of peripheral blood samples after initiation of IM treatment, similar to that reported by other researchers [19].

In some cases, and in order to evaluate the hypocellular patients with peripheral blood sample of low quality, cytogenetic response was calculated with at least ten metaphases [20]. It was reported that if there were fewer than 20 metaphases, the cytogenetic response can be validated using other conformational tests as PCR or FISH techniques [21, 22, 23, 24, 25]. Other reasons behind cultures failure may be related to the physiological differences in cells cycle between patients, or may be the malignant cells were divided very rapidly or not divided when cultured so, metaphases can not be obtained .Also, an inhibiter materials, other than drug, that interferes with cell division could be present. Other reasons could be attributed to responsiveness of cultured cells to the phytohemeagglutinin (PHA), the inducer material for lymphocytes division. It was reported that lymphocytes responsiveness to PHA differs from patients to patients. Other factors or variants involved in cytogenetic culture successfulness include: patient's age, gender, general health and all of these may be due to the type of mutations that occurred in the genetic material of patients and its location [26, 27].

Cytogenetic response and CML Phase

In this study, the percentage of CML patients who achieved MCyR was (64.28 %), approximately similar to what reported by Martinelli *et al*[28], (61%), but it was less than that reported by Guilhot *et al*[29] (78%). Also, a high statistically significant relationship was seen between the degree of cytogenetic response and CML patient's phase, in which (100%) of patients who achieved MCyR were in CP. When we compared between the rates of cytogenetic response (MCyR or PCyR) during different IM treatment durations, it seems that the highest percentage of patients who achieved MCyR seen after (6, 12 and24) ms in IM treatment were approximately stable, but after that (≥36ms) the rate of MCyR started to decrease. That is consistent with the observation of Martinelli *et al*[28], who reported that the rate of MCyR did not consistently increase after 24ms of IM treatment.

Patients who achieved only PCyR seem to have more affinity to progress to advanced phase than those who achieved MCyR. As seen from the results, six patients who achieved only PCyR after a mean of IM treatment duration (35.4) ms were at CP when enrolled in this study, but they were transformed to AP. It was referred to that CP-CML patients who achieved MCyR have significantly lower risk of progression in the subsequent 24ms than patients not achieving MCyR [29, 30, 31]. It was reported that, 97% of patients with a complete cytogenetic response within 12 ms after starting IM did not progress to the accelerated phase or blast crisis by 60 ms. Notably, patients who were deemed to be at high risk on the basis of Sokal scores had a lower rate of complete cytogenetic response (69%) than did patients who were at low risk or intermediate risk (89% and 82%, respectively). However, the risk of relapse in patients who had a cytogenetic response was not associated with the Sokal score. With interferon treatment, by contrast, the Sokal score was important even among patients with a complete cytogenetic response [33].

Obviously, therapeutic decisions would be greatly facilitated if it was possible to accurately predict cytogenetic or molecular response prior to imatinib therapy. Although several baseline factors predictive of subsequent MCyR have been identified, these variables are of little use for decision-making in individual patient [30].

Classification according to the Sokal score defines 3 distinct risk groups but other factors must be taken into account. For example, patients with deletions flanking the ABL or BCR breakpoint have lower rates of CCyR and a shorter time to progression [34].

CONCLUSIONS:

Conventional cytogenetic karyotyping is necessary for Ph–chromosome detection and also, as an assay for periodical assessment of cytogenetic response in CML patients treated with imatinib. Imatinib has resulted in cytogenetic responses in first line IM treated patients and in those who have failed previous IFN-α therapy and in CML patients at early and late chronic phase.

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