Chronic Myelogenous Leukemia: Cytogenetic and Biochemical Consequences and Applications for Diagnosis and Judgment

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Abstract

Background: Chronic myelogenous leukemia (CML) is a clonally myeloproliferative disorder of the hematopoietic stem cell. Arginase activity is high during the mitotic cycle. In addition, TGF-β1 is one of cytokines that responsible for immune cell dysfunction in patients with cancer.

Methods: Blood samples from six diagnosed CML cases studied and compared with control subjects. The first three CML cases were in accelerated –phase (AP)-CML which, resistant to chemotherapy. The other three cases responded for the treatment. Cytokinesis Blocked Micronucleus (CBMN) assay, arginase and TGF-β1 levels were estimated for each CML and control groups.

Results and Conclusion: The treatment resistant group is characterized by low incidences of binucleated and necrotic cells and low micronuclei expressions. Whereas, high frequencies of nucleoplasmic bridges (NPBs) were scored (anaphase nucleoplasmic bridges). Increased levels of arginase and TGF-β1 were recorded in the treatment resistant group when compared with control and treated groups. The resistant cases characterized by low incidences of binucleated cells and micronuclei and high count of NPBs explained by the high rate of mitotic division. Whereas, the levels of arginase and TGF-β1 were increased in the resistant cases in comparison with those of treatment responded and control groups.

Cytokinesis Blocked Micronucleus assay designed as diagnostic tool for differentiation between responding or resistance chemotherapy in CML cases. Arginase, TGF-β1 levels recorded highly significant rules for the same object.

Keywords: Arginase; Blood culture; CBMN assay; CML; TGF- β1

Introduction

CML has a triphasic clinical course: an initial indolent chronic phase (CP), which is present at the time of diagnosis in ~83% of patients with a median duration of 3-5 years; an accelerated phase lasting 6 to 18 months, in which neutrophil differentiation becomes progressively impaired and leukocytic counts are more difficult to control with myelosuppressive medications; and a terminal blast crisis (BC), a condition resembling acute leukemia lasting 3 to 6 months in which myeloid or lymphoid blasts fail to differentiate [1].

CML is characterized by the Philadelphia chromosome (Ph), which represents a reciprocal translocation between the long arms of chromosome 9 and 22, t (9; 22) (q34: q 11) which forms and creates a novel fusion gene BCR-ABL. the Ph links the BCR of chromosome 22 with the ABL proto-oncogene of chromosome 9. The normal ABL gene product is a tightly regulated tyrosine kinase involved in cell division and apoptosis. The BCR-ABL fusion gene product is a constitutively active tyrosine kinase, the presence of which seems sufficient to induce leukemia in both experimental animals and humans [2].

Nilotinib (Tasgina) is pharmacologically related to imatinib mesylate (Gleevec) and dasatinib (Sprycel), which are inhibitors of BCR-ABL tyrosine kinase. Imatinib resistance can be defined as lack of complete hematologic response in patients with CP-CML, or as a failure to return to CP for patients with CML in AP or BC. The majority of patients with imatinib-resistant CML either have secondary BCR-ABL mutations that impair the ability of kinase to adopt the closed conformation to which imatinib binds or directly interfere with drug binding. Drug resistance is associated with reactivation of BCR-ABL signal transduction [3,4].

Chromosomal aberrations can occur spontaneously or after exposure to genotoxic agents and play an important role in cancer pathogenesis [5]. There is considerable interest in understanding the mechanisms underlying acquisition of chromosomal aberrations in leukemic progenitor cells. CML is a prototypical stem cell malignancy with a natural course of progression from CP to AP and BC. Progression to BC is associated with acquisition of additional chromosomal aberration beyond the underlying t (9; 22) chromosomal translocation that characterizes CML [6]. The primary abnormality in CML, the BCR-ABL oncogene, may induce genomic instability that can predispose cells to additional mutations. BCR-ABL may enhance production of reactive oxygen species (ROS) resulting in enhanced
endogenous DNA damage. CML cells may also have altered DNA repair process, including error-prone homologous recombination (HR) and non-homologous end joining (NHEJ) mechanisms [7,8]. However, the relationship of these abnormalities to chromosomal instability CML cells is less well studied [9].

Over the past 17 years, the CBMN assay has evolved a comprehensive method for measuring breakage, DNA misrepair, chromosome loss, non-disjunction, necrosis, apoptosis and cytostasis [10,11]. This method now also used to measure nucleoplasmic bridges (NPBs), a biomarker of dicentric chromosomes resulting from telomere end-fusions or DNA misrepair [12]. The significance of these developments and the concept of the CBMN assay as a ‘Cytome’ assay which implies that every cell in the system studied is scored cytologically for its viability status (necrosis and apoptosis), its mitotic status (mononucleated, binucleated and multinucleated) and its chromosomal damage or instability status (presence of MNi, NPBs). For these reasons, it is now appropriate to refer to this technique as the cytokinesis block MN cytome (CBMN cyt.) assay [12].

Arginase (L-arginine amidinohydrolase) which catalyzes the hydrolysis of L-arginine into L-ornithine and urea was first detected in mammalian livers as the terminal enzyme of the urea cycle [13]. Arginase activity occurs also in other tissues, which are devoid of a complete urea cycle [14]. In the latter instance, the importance of arginase may be in the production of ornithine for the synthesis of the polyamines putrescine and spermine, which are required for normal cellular proliferation. Several reports indicate that a higher activity of arginase is present in cancerous tissues which differ from normal tissues [15].

There are clear relations between mitotic disorders, chromosomal aberrations, CBMN assay, arginase and TGF-β family. Mussai et al. [16] stated that for the first time AML blasts alter the immune microenvironment through enhanced arginine metabolism. Arginase II is expressed and released from AML blasts and is present at high concentrations in the plasma of patients with AML, resulting in suppression of T-cell proliferation. They extended these results by demonstrating an arginase-dependent ability of AML blasts to polarize surrounding monocytes into a suppressive M2-like phenotype in vitro and in engrafted no obese diabetic–severe combined immunodeficiency mice. In addition, AML blasts can suppress the proliferation and differentiation of murine granulocyte-monocyte progenitors and human CD34+ progenitors. Finally, they showed that the immunosuppressive activity of AML blasts can be modulated through small molecule inhibitors of arginase and inductive nitric oxide synthase, suggesting a novel therapeutic target in AML. Pancytopenia observed at diagnosis. In addition, polyamine depletion by deprivation of ornithine and polyamines causes chromosomal damage in the mammalian cells [17].

The TGF-β family is a part of a super family of proteins known as the transforming growth factor beta, which includes inhibins, activin, and bone morphogenetic protein. TGF-β acts as antiproliferative factor in normal cells and at early stages of oncogenesis [18,19]. In normal cells, TGF-β, acting through its signaling pathway, stops the cell cycle at the G1 stage to stop proliferation, induces differentiation, or promotes apoptosis. When a cancer cell, parts of the TGF-β signaling are mutated, and TGF-β no longer controls the cell [20].

On the other hand, Tumorgenesis in rodents, as well as in humans, has been shown to be a multistep process, with each step reflecting an altered gene product or gene regulatory process leading to autonomy of cell growth. Initial genetic mutations are often associated with dysfunctional growth regulation, as is demonstrated in several transgenic mouse models. These changes are often followed by alterations in tumor suppressor gene function, allowing unchecked cell cycle progression and, by genomic instability, additional genetic mutations responsible for tumor metastasis.

Here we show that reduced transforming growth factor-factor-β signaling in T lymphocytes leads to a rapid expansion of a CD8+ memory T-cell population and a subsequent transformation to leukemia/lymphoma as shown by multiple criteria, including peripheral blood cell counts, histology, T-cell receptor monoclonality, and host transferability. Furthermore, spectral karyotype analysis of the tumors shows that the tumors have various chromosomal aberrations. These results suggest that reduced transforming growth factor-factor-β signaling acts as a primary carcinogenic event, allowing uncontrolled proliferation with consequent accumulation of genetic defects and leukemic transformation. TGF-β family members are a multifunctional group of secreted proteins that function to control growth, differentiation, and cell death. TGF-β signals are complex in nature and exert different effects depending on cell type, environment, and subsequent signaling pathways. When interacting with epithelial and hematopoietic cells, two cell types that give rise to many cancers, TGF-β signaling becomes inhibitory, thus acting as a tumor suppressor in the early stage of carcinogenesis [21].

The purpose of this study was to explore characteristic features of CML using novel tools such as CBMN cytoe assay, arginase and TGF-β. Moreover, the presented work discusses the difference between the resistance and responding achievement to nilotinib drugs depending on the clinical investigation.

Materials and Methods

Chemicals

The chemicals of the blood culture were purchased from GIBCO-BRL, USA, heat-inactivated foetal calf serum (FCS) from Sigma-Aldrich chemical Co, St Louis, USA, TGF-β1 ELISA kit from DRG International, Inc. USA, Corp@drg-international.com, and arginase from Bio-diagnostic Comp., biodiagnostic_eka@lycos.com

Blood sampling and experimental design

Blood samples obtained from six cases of CML patients, three of them were diagnosed as AP-CML that were drug resistant. The others were CP-CML, responded to the drug treatment. The diagnosis was based on clinical examination and laboratory evaluation, which carried out by the consultant medical staff. All the cases were clinically diagnosed with a medium duration 1-2 years at the accelerated phase. We chose three cases were responded to nilotinib and returned to the CML, others three cases were not responding through the same period of treatment and still persisted in AP. One patient from this group evolved to death.

The mean value of WBCs count for the T-group at the beginning of treatment was 80,000 cells/mm³ and within 8 to 12 months through the nilotinib period reached to 8,000 cells/mm³. In addition the Bcr-abl was decreased through the same period of the treatment from the ratio 2.03±0.27 prior to sampling, whereas the TR-group recorded the following data: mean value of total leukocytes count at the beginning of the treatment was 118,000 cells/mm³ and within one year of nilotinib treatment, the mean value was 90,000 cells/mm³ (Bcr-abl
Determination of TGF-B1 cells and low expression for the MNi. In addition, this group were matched for ethnic background, sex, smoking and age.

All subjects were gave an informed consent for participation in the study. The donors were selected according to current International Programme on Chemical Safety (IPCS) guidelines for the monitoring of genotoxic effects of carcinogens in human [22].

Venous blood were collected under sterile conditions in heparinized vacationer tube (V= 5 ml), (Becton Dickinson, USA) containing Lithium heparin as anticoagulant.

The heparinized blood from each subject divided into two parts, the first for culture set-up in triplicate and the second for the biochemical analysis using two ways analysis of variance "F" test according to Abramowitz and Stegum [25], the level for statistical significance was p<0.05.

Blood culture

Blood cultures were set up for 72 hrs according to the protocol described by Evans and O’ Riodran [23] and its modification by Fenech [12].

Cytokinesis-block Micronucleus (CBMN) Assay

CBMN assay performed as described by Fenech [10,12] which referred as cytome assay. We recorded mono-, bi-, tri- and quadrinucleated cells and cytotoxicity via necrotic and/or apoptic cell ratio. Moreover, MNi and NPBs frequencies in the cells were detected for 1000 cells in each sample.

Determination of arginase

The method used by Biodiagnostic Company for plasma based upon the colorimetric determination of urea by condensation with diacetylmonoxime in an acid medium in the presence of ferric chloride (oxidant) and carbazide (accelerator) [24].

Determination of TGF-B1

TGF-B1 was determined according to the method described by DRG, TGF-B1 ELISA kit (DRG International. Inc. USA) based on the sandwich principle.

Statistical Analysis

Data were present as distribution analysis, percentages, means ± SE and analyzed using two ways analysis of variance "F" test according to Abramowitz and Stegum [25], the level for statistical significance was p<0.05.

Results

Table 1 showed that TR-group had low incidence of binucleated cells and low expression for the MNi. In addition, this group characterized by high percentages of NPBs with unique form (multinucleated thread-like bridges) and mononucleated cells as shown in Figure 3A, 3B, 3H and 31.

T-group data scored high values comparing to TR-group in the frequencies of binucleated cells (1.8 fold), MNi in mononucleated cells (6.5 folds) and binucleated cells (3.7 folds) Figure (2A-2D, 2K and 2L). On the other hand, in T-group, the count of mononucleated cells was decreased (1.4 fold), but the count of the necrotic cells was increased (5.7 folds) when compared with TR-group (Figure 2E, 2L 2J and 3C, 3J). The NPBs were decreased significantly in T-group with 8.3 folds when compared with TR-group as shown in Figure 3E and 3J.

| Groups | C-group | TR-group | T-group |
|--------|---------|----------|---------|
| %      | X ± S.E | X ± S.E  | X ± S.E |
| Mononucleated cells | 64.17 ± 15.06 | 77.66 ± 9.68a | 55.16 ± 15.06 |
| Mononucleated cells + 1 Mn | 0.45 ± 0.92 | 9.5 ± 2.84a | 6.16 ± 0.66 |
| Mononucleated cells + 2 Mn | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.05 ± 0.0 |
| Binucleated cells | 32.4 ± 11 | 9.62 ± 12.77a | 17.60 ± 16 |
| Binucleated cells + 1 Mn | 0.67 ± 0.26 | 6.03 ± 1.32 | 2.21 ± 2.21 |
| Binucleated cells + 2 Mn | 0.0 ± 0.0 | 0.40 ± 0.33 | 0.0 ± 0.0 |
| Nucleoplasmic bridges | 0.16 ± 0.58 | 5.24 ± 5.24 | 0.83 ± 0.30b |
| Trinucleated cells | 1.01 ± 4.15 | 0.77 ± 1.98 | 0.30 ± 3.0 ± 1.28 |
| Quadrinucleated cells | 0.37 ± 2.39 | 0.24 ± 1.33 | 0 ± 1.13 |
| Apoptic cells | 0.41 ± 0.83 | 2.10 ± 2.68a | 2.06 ± 1.34 |
| Necrotic cells | 0.14 ± 0.60 | 2.77 ± 9.03a | 15.83 ± 15.83 |

Results represents as mean ± standard error
C-group: Control group
TR: Treatment resistant group
T: Treated group.
*p < 0.05
a: P-value Significant when compared with C- group.
b: P-value Significant when compared with TR-group.

Table 1: The incidence of mono-, bi-, tri-, quadrinucleated, apoptic, necrotic cells, and the frequencies of micronuclei and the nucleoplasmic bridges in CML and control groups.(counts in 1000 cells).

Whereas, the statistical difference between the two groups was non-significant when compared for the counts of apoptic cells (Figure 2F-2H and 3C). In addition, the results showed that there were not significant differences between the counts of trinucleated and binucleated cells for all groups (Figure 2M, 3D, 3F and 3G), but C-group recorded the highest counts. C-group data presented the ratios of binucleated cells (32.4%) and mononucleated cells (64.1%).
Besides, it was characterized by low frequencies of MNi, NPBs, apoptic cells and necrotic cells Figure 1A-1C.

Table 2: The frequencies of total numbers of mono-, binucleated cells with and/or without micronuclei, and the total numbers of aberrant cells.

| Groups                          | C-group % (X ± S.E.) | TR-group % (X ± S.E.) | T-group % (X ± S.E.) |
|--------------------------------|----------------------|-----------------------|----------------------|
| Total no. of mononucleated cells with and/or without Mn | 64.62 (646.2 ± 16.76) | 78.61 (786.1 ± 9.29) | 61.37 (613.7 ± 27.73) |
| Total no. of binucleated cells with and/or without Mn & NB | 33.24 (332.4 ± 10.74) | 15.50 (155 ± 5.27) | 20.43 (204.3 ± 38.54) |
| Total no. of aberrant cells (cells with Mn, NB & necrotic, apoptic, trinucleated, quadrinucleated cells) | 3.63 (36.3 ± 4.15) | 12.72 (127.2 ± 22.03) | 25.24 (252.4 ± 55.42) |

Results represents as mean ± standard error
C-group: Control group
TR: Treatment resistant group
T: Treated group
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a: P-value Significant when compared with C-group
b: P-value Significant when compared with TR-group

Table 3 showed the results of the biochemical investigations for the three groups. Arginase level was higher in TR-group than T-group with 1.7 folds and more than C-group with 3.9 folds. On the other hand, TGF-β1 value of the TR-group was insignificantly higher than that of T-group. Both groups had significant increment of TGF-β1 levels when compared with C-group (3.9-4.1 folds) Figure 4.

Table 3: The levels of Arginase and TGF-β1 in human CML and control groups.

| Groups                          | Arginase (IU/L) | TGF-β1 (pg/ml) |
|--------------------------------|----------------|----------------|
| C-group | 110.27 ± 2.15 | 1336.70 ± 144.10 |
| TR-group | 439.44 ± 29.82a | 5500.00 ± 124.87a |
| T-group | 256.58 ± 33.24ab | 5233.30 ± 45.84a |

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discussion

Allogeneic stem cell transplantation was the first treatment modality in CP-CML that was capable of inducing of cytogenetic and molecular remissions and resulted in superior long-term leukemia-free survival in approximately 50% of patients [26].

In the early 1980s, it was demonstrated that interferon-α induced complete or partial cytogenetic remission in approximately 20% of patients with CP-CML. Therefore, some investigators appropriately argued at the time that patients with CP-CML could undergo a treatment trial with interferon-α first and could consider transplantation only if they failed to achieve complete cytogenetic remission [27].

Recently, nilotinib and imatinib have replaced both allogeneic stem cell transplantation and interferon-α as first-line treatment for CP-CML. Nilotinib and imatinib are the generation of tyrosine kinase inhibitors of c-ABL oncogene I receptor tyrosine kinase, arginase, platelet derived growth factor receptor and they target the adenine triphosphate binding site within the breakpoint cluster region (BCR)-ABL-1 fusion protein, which is the disease-causing mutant kinase in CML [2,28].
Figure 2: Micrographs of CML cells of T-group (A-M).

Figure 3: Micrographs of CML cells of TR-group (A-J).
The follow-up of patients who were randomized to the imatinib or nilotinib study indicated that the overall survival rate was 88%. There are some cases that were resistant or intolerant of imatinib or nilotinib, and they have secondary BCR-ABL mutation that interfered with the drug binding and this associated with the reactivation of BCR-ABL signal transduction [29].

The CBMN cytome assay is a comprehensive system for measuring DNA damage, cytostasis, and cytotoxicity. DNA damage events are scored specifically in once-divided binucleated cells and include (a) MN, a biomarker of chromosome breakage and/or whole chromosome loss, (b) NPBs, a biomarker of DNA misrepair and/or telomere end-fusion. Fenech (2007) stated that the cytokinesis-block micronucleus cytome assay is a comprehensive system for measuring DNA damage, cytostasis and cytotoxicity. DNA damage events are scored specifically in once-divided binucleated (BN) cells and include (a) micronuclei (MNi), a biomarker of chromosome breakage and/or whole chromosome loss, (b) nucleoplasmic bridges (NPBs), a biomarker of DNA misrepair and/or telomere end-fusions, and (c) nuclear buds (NBUDs), a biomarker of elimination of amplified DNA and/or DNA repair complexes. Cytostatic effects are measured via the proportion of mono-, bi- and multinucleated cells and cytotoxicity via necrotic and/or apoptotic cell ratios. Further information regarding mechanisms leading to MNi, NPBs and NBUDs formation is obtained using centromere and/or telomere probes. The assay is being applied successfully for biomonitoring of in vivo genotoxic exposure, in vitro genotoxicity testing and in diverse research fields such as nutrigenomics and pharmacogenomics as well as a predictor of normal tissue and tumor radiation sensitivity and cancer risk. The procedure can take up to 5 days to complete [12].

Fenech [12] and Hoffelder et al. [30] proposed that NPBs between nuclei in BN cells should be scored in the CBMN assay because they provide a measure of chromosome rearrangement. NPBs occur when centromeres of dicentric chromosomes are pulled to opposite poles of the cell at anaphase. It is possible to observe dicentric anaphase bridges before the nuclear membrane is formed, because cells proceed through anaphase and telophase rapidly, completing cytokinesis, which ultimately results in breakage of the NPB when the daughter cells separate. However, in the CBMN assay, BN cells with NPBs are allowed to accumulate because cytokinesis is inhibited and the nuclear membrane is eventually formed around the chromosomes allowing an anaphase bridge to be observed as NPB. Furthermore, chromosomal instability is a key step in the generation of the cancer cell karyotype. An indicator of unstable chromosomes is the presence of chromatin bridges during anaphase. We examined in detail the fate of anaphase bridges in cultured oral squamous cell carcinoma cells in real-time. Surprisingly, chromosomes in bridges typically resolve by breaking into multiple fragments. Often these fragments give rise to micronuclei (MN) at the end of mitosis. The formation of MN is shown to have important consequences for the cell. We found that MN have incomplete nuclear pore complex (NPC) formation and nuclear import defects and the chromatin within has greatly reduced transcriptional activity. Thus, a major consequence of the presence of anaphase bridges is the regular sequestration of chromatin into...
genetically inert MN. This represents another source of ongoing genetic instability in cancer cells.

In the present study, the results indicated that TR-group had some characteristic data which different from T-group. Such as, low frequencies of binucleated cells, decreased levels of MNi expressions, and low incidence of apoptotic and necrotic cells. While, the NPBs scored with high ratio in TR-group when compared with T-group, moreover the NPBs characterized in this study with unique form ( multinucleated thread-like shape) that different from that of C-group and T-group.

These results discussed in view of the CBMN cytome assay by Fenech (12).The blood progenitor cells of the TR-group have a very high rate of mitotic division that cannot arrested by cytochalasine B. So, the cells divided more than one time in blood culture. This event occurred in the presence of unbalanced aberration, translocation and complex karyotypes which led to abnormal NPBs, low incidences of apoptosis, necrosis and increasing in the count of the blood progenitor cells. The previous findings agreed and consistent with those of Brady et al. [31] and Nakanashi et al. [32].

Various mechanisms can led to NPBs formation following DNA misrepair of strand breaks in DNA [33]. Typically, a dicentric chromosome and an acentric chromosome fragments are formed and caused the development of NPBs and MNi, respectively. Misrepair of DNA strand breaks could also lead to the formation of dicentric ring chromosome and concatenated ring chromosome which could also result in the formation of NPBs. An alternative mechanism for dicentric chromosome and NPBs formation is telomere end fusion caused by telomere shortening, loss of telomere capping proteins or defects in telomere cohesion [34].

Table 2 showed that the comparison between the groups for the total counts of mono-and binucleated cells which indicated that TR-group less responded to arresting mitosis than in T- and C-groups. The apoptosis and the necrosis processes were recorded in T-group group less responded to arresting mitosis than in T- and C-groups.

The investigation on the pattern of distribution of enzymes in different tissues is of particular importance as this type of information can help localize certain biochemical processes that are unique to a tissue. In addition, such information might provide a basis for developing diagnosis and therapeutic approaches when these tissues are damaged or encountered a malignant state.

Results obtained in the study (Table 3) are consistent with this notion. Arginase was recorded in TR-group with high level more than T-group, so the total count of aberrant cells for T-group was observed more than TR- and C-groups.

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On the other hand, the level of TGF-β1 in TR-group was insignificantly increase more than T-group and C-group. TGF-β1 is a protein that controls proliferation, cellular differentiation, and other functions in most cells. It is a type of cytokine which plays a role in immunity, cancer, bronchial asthma, heart disease and diabetes [38]. TGF-β acts as an antiproliferative factor in normal cells and at early stages of oncogenesis, in addition TGF-β1 induces apoptosis in numerous cell types and plays a crucial role in the regulation of the cell cycle [19].

There is high expression of genes related to drug resistance and inhibiting cell apoptosis. In order to address the mechanism of CD133 positive tumor cells showing strong resistance to therapeutic drugs, both CD133 positive cells and CD133 negative cells were collected to investigate the expression of multi drug resistance and DNA mismatch repair related genes, as well as related genes related to inhibiting cell apoptosis within these two populations. BCRIPI has been demonstrated to play an important role in the drug resistance of normal stem cells and tumor stem cells [39,40]. In addition, the presence of DNA repair protein MGMT has been demonstrated to render cells resistant to cytotoxic actions of methylating and chloroethylylating agents, such as temozolomide [41,42].

The data presented in this study displayed the importance of CBMN assay as a tool of investigation of the CML-patient to evaluate the cytogenetic instability and the cytogenetic remission with any treatment. In addition, we recommended that the nilotinib and/or imatinib intolerant or resistant for some cases must be supported by mitotic arresting and apoptotic enhancing agents. Moreover, the presence of low incidence of binucleated cells for CML in CBMN assay does not mean low nuclear division index, but it means that the controlling and governing of cytochalasine B for the mitotic division is very high (low division rate).

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Future studies

The resistant cases for chemical treatment should sustained with certain type of radio-therapeutic for enhancement of the apoptic process and management of the high rate of the mitotic division.

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