The role of cytogenetics and molecular biology in the diagnosis, treatment and monitoring of patients with chronic myeloid leukemia

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Chronic myeloid leukemia (CML) is the most common myeloproliferative disorder among chronic neoplasms. The history of this disease joins with the development of cytogenetic analysis techniques in human. CML was the first cancer to be associated with a recurrent chromosomal alteration, a reciprocal translocation between the long arms of chromosomes 9 and 22 – Philadelphia chromosome. This work is an updated review on CML, which highlights the importance of cytogenetics analysis in the continuous monitoring and therapeutic orientation of this disease. The search for scientific articles was carried out in the PubMed electronic database, using the descriptors “leukemia”, “chronic myeloid leukemia”, “treatment”, “diagnosis”, “karyotype” and “cytogenetics”. Specialized books and websites were also included. Detailed cytogenetic and molecular monitoring can assist in choosing the most effective drug for each patient, optimizing the treatment. Cytogenetics plays a key role in the detection of chromosomal abnormalities associated with malignancies, as well as the characterization of new alterations that allow more research and increase knowledge about the genetic aspects of these diseases. The development of new drugs, through the understanding of the molecular mechanisms involved, will allow a possible improvement in the survival of these patients.

Key words: BCR-ABL positive chronic myeloid leukemia; leukemia; cytogenetics; karyotype; Philadelphia chromosome; therapeutics.

INTRODUCTION

Chronic myeloid leukemia (CML) is a rare type of neoplasia, with incidence of 1-2 cases per 100,000 individuals annually. CML is the most common chronic myeloproliferative neoplasms, representing 0.5% of all new cancer cases in the United States. This represents 15% of all leukemia in adults and is more common in men than women (1.3-1.7 compared to 1.0). The National Institutes of Health (NIH) estimates that, in 2017, there will be 8,950 new cases of CML and approximately 1,080 people will die from this disease. CML is most frequently diagnosed among individuals with 40-60 years of age, with mean age of 53 years and only 10% of patients are diagnosed with less than 20 years of age.

CML is characterized by clonal expansion of hematopoietic progenitor cells, resulting in increased circulating cells of granulocytic lineage. The characteristic symptoms of this disease are chronic fatigue, weight loss, bleeding and fever; whereas signals are anemia, granulocytosis, and immature granulocytes, presence of basophils, thrombocytosis and splenomegaly. The first treatments for CML provided a significant improvement in the quality of life at the chronic phase disease. For several years, the only treatment available for patients was hematopoietic stem cells transplantation. The development of cytogenetic research involving the Philadelphia chromosome and molecular alterations enabled the emergence of a new perspective for the CML treatment, making it more accurate and effective. The aim of this article is an updated review about CML, highlighting...
the importance of cytogenetic analysis for monitoring progress and therapeutic management of this disease.

CML AND CYTOGENETICS

Cytogenetics is a crucial tool for the diagnosis, prognosis and treatment of neoplasms, such as CML. The knowledge about this disease arose along with the development of analytical techniques in human cytogenetics. CML was the first identified recurrent cancer-associated chromosome change, the Philadelphia chromosome or Ph, marker of CML. In 1960, Nowell and Hungerford identified a small chromosome present at high frequency in cells from CML patients, an event confirmed by other researchers in the field of oncology. These cells had a normal number of chromosomes, but one of these chromosomes was noticeably smaller when compared to the others.

For decades, it was believed that the Ph chromosome formation was a result of deoxyribonucleic acid (DNA) loss by simple deletion. However, the chromosome banding technique changed this perspective. In 1972, Janet Rowley found that the Ph chromosome was the result of a reciprocal translocation between the long arms of chromosomes 9 and 22, in which a large portion of chromosome 22 was transferred to chromosome 9, and a small portion of chromosome 9 was transferred to 22, whereby the Ph chromosome becomes smaller than the normal chromosome 22.

After years, it was possible to have an accurate identification of the breakpoints of the chromosomes involved in the Ph chromosome translocation and the understanding of gene structure by cytogenetics and molecular biology. It was found that Abelson murine leukemia viral oncogene, also known as ABL1, located on the long arm of chromosome 9 at 9q34.12, is usually broken within the intron 1. However, this breakpoint can occur anywhere over a large (300 kb) area at its 5'-end, either upstream of the first alternative exon 1b, downstream of the second alternative exon 1a, or, more frequently, between both. The breakpoint cluster region (BCR) gene is located on the long arm of chromosome 22 at 22q11.23 and has three breakpoints: minor breakpoint cluster region (M-bcr), major breakpoint cluster region (M-bcr) and micro breakpoint cluster region (μ-bcr). The fusion of these genes occurs at the 5' end of the ABL and the 5' end of the BCR gene. The fragments of the BCR and ABL1 genes resulted a chimeric derivative chromosome 22 or Ph chromosome. The hybrid gene resulting from this fusion, named BCR-ABL1, encodes a chimeric protein with new oncogenic properties. This results in an increase of tyrosine kinase activity, such as induction of cellular proliferation, malignant transformation of immature granulocytes and lymphocytes, and apoptosis suppression. Moreover, it also affects stem cell differentiation and DNA repair. Ph chromosome-positive cells are genetically unstable and likely to develop several genonic abnormalities, resulting in a progression from chronic phase (CP) to accelerated phase (AP) and blast phases (blast crisis (BC)).

The cytogenetic techniques and new methodologies associated with molecular biology techniques were optimized over time. Methodologies as fluorescence in situ hybridization (FISH), spectral karyotyping (SKY), chromosome painting probes, and microarrays-based comparative genomic hybridization (array-CGH) are applied in the clinical research and diagnosis of patients with developing cancer. Through these techniques, the chromosomes can be analyzed accurately and precisely, exceeding the resolution of the conventional cytogenetic analysis, which detects only abnormalities larger than 5 Mb, depending on the altered chromosomal region. Thus, it was possible to discover new chromosomal abnormalities associated with different types of leukemia, especially involving complex changes. Currently, about 1,000 balanced chromosome rearrangements and 600 gene fusions cancer-related have already been identified.

Chromosomal variants of CML

CML is classically characterized by the presence of the Ph chromosome (Ph+), found in 90% of the patients. In the absence of Ph chromosome (Ph-), CML is considered atypical (aCML) and pathobiologically distinct from the classic CML. This kind of leukemia is extremely rare, with an incidence of 1-2 cases per 100 cases of Ph+ CML. The rates of survival and survival without disease progression are estimated at 80% and 86%, respectively.

Beyond simple reciprocal translocation between chromosome 9 and 22, about 5%-10% of CML cases may have variant Ph translocations. These simple variants involve one or more chromosomes in addition to 9 and 22; always changing chromosome 22. Secondary changes or other complex cryptic chromosomal rearrangements in BCR-ABL1 may be observed in 9%-16% of CML patients with normal karyotype.

Frequently, additional chromosomal abnormalities (ACAs) are found in Ph+ cells and are classified into “major” and “minor” route changes. These alterations interfere with the progression of the disease and increase in the advanced stage, from 30% in AP to 80% in BC. They are related to poor prognosis, with lower rate of cytogenetic response rate to treatment with imatinib mesylate (IM). Major-route ACAs are more associated with worse prognosis.
than minor-route ACAs, highlighting that the impact of cytogenetic
alterations depends on the type of ACA regardless of the percentage
that these changes occur\(^{20}\). The most frequent abnormalities
detected in advanced CML with major-route ACA are trisomy 8,
isochromosome 17q and second Ph chromosome. They are related
to shorter overall survival and progression-free survival. Minor-
route ACAs are rarely observed in AP or BC, including t(1;21),
t(1;22), t(2;16), t(3;12), and t(4;16)\(^{20}\).

Reciprocal rearrangements considered as minor route
involving 11q23 and 3q26.2, extremely rare in CML, have been
described. These patients have low response rate to treatment with
tyrosine kinase inhibitor (TKI) and poor clinical outcome\(^{21, 22}\).
Although not completely clear, this information may help the
physician to decide on therapeutic alternatives, since the presence
of these abnormalities during treatment reflect in genetic
instability and consequently a negative response to the drug\(^{17}\).

**Cytogenetics and molecular analysis**

Conventionally, a 24 to 48-hour bone marrow cells culture
is performed for chromosome analysis from patients with CML.
Then, colchicine is added, a drug that prevents achromatic spindle
formation by keeping the cells in metaphase, the point at which the
chromosomes are more condensed. The cells undergo hypotonic
shock (potassium chloride) and are washed with a fixating solution
(acetic acid and methanol). The material is placed on glass slides
and stained conventionally using chromosome banding with
trypsin and Giemsa (GTG banding). At least 20 metaphases must be
analyzed under the microscope; 30%-50% of chromosomal variants
in CML may present fused genes that can be detected by molecular
techniques such as FISH or polymerase chain reaction (PCR)\(^{19, 20}\).

In addition to the Ph translocation t(9;22), other
chromosomal aberrations can be detected through chromosome
banding. FISH and PCR are able to detect rare BCR-ABL variants
and breakpoints that go unnoticed by conventional cytogenetics,
with higher specificity and sensitivity, besides a rapid diagnosis.
These techniques are more sensitive and important not only for
diagnosis and evaluation of response to treatment, but also for
the differentiation from other myeloproliferative diseases. FISH
is recommended for diagnosis in cases where the Ph chromosome
is not detected by classical cytogenetics. Techniques such as real-
time quantitative PCR (RQ-PCR) or reverse transcription-PCR
(RT-PCR) are the method of choice for detection of minimal
residual disease after bone marrow transplantation due to their
greater sensitivity, allowing early diagnosis of disease relapse and
more suitable for determining response to treatment than the
classical methods\(^{21}\).

**MOLECULAR CHARACTERIZATION**

Protein tyrosine kinase (PTKs) and signaling pathways are
frequent targets of oncogenic mutations, their role in mitogenic
activity and decrease with apoptosis is finely regulated in normal
cells\(^{2, 3}\). However, in mutant cells or structurally modified cells,
PTKs are constitutively active and becomes powerful oncoproteins,
promoting cellular transformation\(^{20}\). CML is strongly associated
with generation of the BCR-ABL1 fusion oncogene, due this fusion,
the kinase activity of the ABL1 portion is increased promoting
autophosphorylation and triggering a series of functional changes
in important proteins of cellular regulation, such as Ras family,
MAPK, STAT, p38K and MYC, triggering the malignant phenotype
of the disease\(^{20}\). Studies suggest that nuclear ABL1 plays an
important role in the induction of apoptosis and acts as a carrier
between the nucleus and the cytoplasm. BCR fusion generates loss
of function and maintains ABL1 in the cytoplasm assuring its
interaction with the mentioned proteins\(^{26, 27}\).

Although the BCR-ABL1 chimeric protein is widely studied,
the BCR normal function remains unclear. BCR is a broadly
expressed protein that acts on the polymerization of other proteins,
with serine/threonine kinase activity and a GDP-GTP exchange
domain involved in the regulation of members of the Ras family
and guanosine activation of the triphosphate domain for the
regulation of GTPase, Ras and Rho\(^{26}\).

**BCR** gene is a breakpoint that generates two alternative forms
of the Ph chromosome translocation found in CML and acute
lymphoblastic leukemia (ALL)\(^{20}\). In most Ph+ CML patients,
the BCR breakpoint covers exons e12-16 (historically known
as b1-b5) in the M-bcr region. The alternative splicing leads
to fusion transcripts (e13a2 or e14a2), originating a 210 kDa
(p210<sub>BCR-ABL1</sub>) protein responsible for the typical phenotype of CML
individuals\(^{20, 31}\). The p190<sub>BCR-ABL1</sub> protein arising from the m-bcr
rearrangement produces the e1a2 fusion transcript, which is
found in rare cases of CML. Less often, the e19a2 fusion transcript
in µ-bcr (p230<sub>BCR-ABL1</sub> protein) is associated, not in all cases,
with rare Ph+ chronic neutrophilic leukemia of slow disease
progression\(^{32, 33}\). The ABL1 breakpoint usually occurs in the 5'
of exon 2 toward the centromere. From this point, the exons a2
and a11 of the ABL1 fragment are translocated in the BCR gene
(Figure). The ABL1 sequence is almost constant, varying only the
BCR breakpoint that defines the BCR-ABL1 isoforms\(^{20}\).

In addition to the constitutive activation of the mitogenic
signaling, the BCR-ABL1 oncogene acts on the proteasome-
mediated degradation and the molecular mechanisms by which
it promotes cell transformation appear to involve tyrosine kinase-
CML PHASES

The course of the disease can occur in three phases based on the number of blasts in blood and bone marrow: CP, AP and BC (Table). The diagnosis is performed in peripheral blood and bone marrow by cytogenetic analysis, hematologic and clinical findings. In the CP phase, clonal proliferation of granulocytes occurs with the presence of cells with differentiation capacity, which makes the disease manageable. During the course of the disease, the leukemic clone loses the ability to differentiate, making it difficult to control the disease, characterizing its progression to AP, and then to BC, or advancing directly to BC. The AP classification criteria are discussed, due to changes in prognosis and the widespread use of TKIs. With the advent of new drugs that circumvent clonal evolution, the tendency for the next few years is that the AP becomes less frequent.

CML TREATMENT

In just over a decade, the drugs used for CML treatment were limited to non-specific agents, such as busulfan, hydroxyurea and interferon-alpha. The use of interferon-alpha resulted in disease regression and improved patient survival. However, it was discontinued due to its high toxicity and the low proportion of patients with complete cytogenetic response.

First-generation drug

The natural course of CML changed dramatically when, in 1996, Druker et al. (1996) developed the TKI, a drug capable of blocking the constant activity of the ABL protein of the BCR-ABL1 protein (3). This drug, called STI-571 or IM (Gleevec), marked the beginning of a new era in cancer treatment in a detailed molecular study that showed efficient results in patients with poor prognosis and suffering from side effects. Ph+ cells have a proliferative advantage over normal cells, but TKI leads to the self-destruction of leukemic clones, reducing tumor proliferation by disrupting an oncogene-dependent pathway, leading to cellular apoptosis.

IM acts by competing with the ATP receptor in the ABL tyrosine kinase domain, preventing the activation of signal transduction cascades. For 10 years, this drug was the standard therapy for CML due to its remarkable activity and median toxicity, including few side effects. Its implementation in the following years caused a drastic reduction in the use of allogeneic stem cell transplants, obtaining a complete cytogenetic response.
TABLE – Summary of clinical, hematology and cytogenetics features of each phase of CML (2, 3, 39, 44-47)

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Chronic phase</th>
<th>Accelerated phase</th>
<th>Blast phase/blast crisis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical features</strong></td>
<td>Asymptomatic or mild symptoms. Fatigue, weight loss and anorexia. Hypermetabolism. Splenomegaly, hepatomegaly and mild lymphadenopathy. Abdominal discomfort and acute pain in the left upper quadrant</td>
<td>Symptomatic patients (not all). Fever, bone pain, bleeding and sweating. Splenomegaly.</td>
<td>Aggressive presentation. Symptoms similar to those of the accelerated phase worsened. Fever, sweats, bone pain, weakness, loss of sense of well-being and splenomegaly. The patient no longer responds to the medication previously used, contributing to a higher risk of malignancy presentation</td>
</tr>
<tr>
<td><strong>Hematologic features</strong></td>
<td>Moderate anemia. Hypercellular bone marrow. Large proportion of basophils, eosinophils, neutrophils, metamyelocytes and myelocytes. Increased number of megakaryocytes. Granule-erythroid ratio above 10:1. Leukocytosis, often above 100,000 cells/mm³. Increased reticulin deposition within bone marrow</td>
<td>Worsening of anemia. Blood or bone marrow blasts percentage of 10%-19%. Basophils in blood ≥ 20%. Persistent thrombocytopenia (&lt; 100 × 10⁹/l) unrelated to therapy. Increased white blood cell count unresponsive to therapy</td>
<td>Blood or bone marrow blasts ≥ 20%. Similar to acute leukemia or presence of organs or tissues infiltration by blasts. Severe anemia, increase in the number of nucleated red blood cells. Bone marrow fibrosis. Decrease in the total number of leukocytes and myelocytes</td>
</tr>
<tr>
<td><strong>Cytogenetic features</strong></td>
<td>Additional abnormalities in positive Ph (excluded Y loss, Ph variants and constitutional abnormalities)</td>
<td>Additional clonal chromosomal abnormalities in positive Ph at diagnosis, including &quot;major route&quot; abnormalities (second Ph, +8, isochromosome 17q, +19), complex karyotype, abnormalities of 3q26.2. Any new clonal chromosomal abnormality in positive Ph that occurs during therapy</td>
<td>Additional abnormalities in positive Ph and Ph abnormalities variants</td>
</tr>
</tbody>
</table>

CML: chronic myeloid leukemia.

in more than 80% of the patients and increasing the long-term survival. The presence of cases of resistance or intolerance to IM has led to the need to develop new inhibitors. This is because the leukemic cells are genetically unstable and susceptible to multiple mutations, which cause the disease to progress (48).

**Second-generation drug**

More than 90 mutations were described in the tyrosine kinase domain, conferring resistance to TKIs treatment. The most common and dangerous mutation occurs due to a replacement of a threonine by leucine to isoleucine at position 315 (T315I). This mutation is resistant to IM and other second-generation drugs. Point mutations in the tyrosine kinase domain of ABL cause conformational changes in the protein making it difficult to link with TKI (10, 49, 50). The TKI second generation molecules, dasatinib, and nilotinib were initially reserved for patients with resistance or intolerance to IM and later recommended to the CML’s CP (33). However, some patients with T315I mutation also show resistance or intolerance to the drugs (49, 52). Random studies have shown that dasatinib and nilotinib are more effective as first-line treatment for CML in CP than IM (43).

The CML’s hematopoietic stem cells (HSC) are resistant to TKIs, surviving even after the BCR-ABL1 blockade. For these cells, there are two possible pathways IM resistance: BCR-ABL1-dependent or -independent. In BCR-ABL1-dependent, there may be amplification or overexpression, gene mutation, clonal evolution or improper administration of the drug. In BCR-ABL1-independent, leukemic cells can develop mechanisms of adaptation and development, explaining the low rates of CML complete remission (53). It is necessary to use the TKI therapy long-term since the drug removal results in the return of BCR-ABL1 expression. Studies showed that HSC does not depend on BCR-ABL1 to survive, unlike mature cells undergoing oncogene-dependent apoptosis (54). Research on the development of other classes of drugs more effective in combating these cells is emerging.

**Third-generation drug**

As an alternative to treatment, the third-generation of TKI emerged: ponatinib (AP24534) and bosutinib. Ponatinib is a third-generation TKI, which has shown to be effective in both early and advanced phases of CML (55, 56). Inhibitors that compete with ATP can be divided into subclasses of Src/ABL inhibitors — dasatinib, bosutinib, AP2464 and PD166326 and subclasses of...
base 2-phenyl amino pyrimidine, such as imatinib, nilotinib and INNO-406. The inhibitors that do not compete with ATP, MK-0457 and ON012380, were developed to inhibit the phosphorylation of mutation T315I of the BCR-ABL1 gene. These drugs aim to avoid IM resistance(42).

Point mutations in BCR-ABL1 confer resistance to imatinib, dasatinib, nilotinib and bosutinib. Ponatinib is the first TKI in class to exhibit activity against CML with the T315I mutation. The mutation known as “gatekeeper” in T315I displays resistance to all currently available TKIs, except ponatinib(57). Drugs BCR-ABL1-independent signaling pathways, such as omacetaxine mepesuccinate, are being tested as a treatment option for patients with previous history of resistance or intolerance to two or more TKI(10, 58). Currently, studies have suggested the use of interferon-alpha combined with TKI(59), as well as HSC transplant for certain patients in AP or BC(5).

The aCML cases are associated with a poor prognosis, requiring differential clinical follow up. Due to the absence of the Ph chromosome and specific somatic mutations, the recommended CML treatments are useless. Specific target therapies have been developed from genetic studies to outline the appropriate treatment. HSC transplantation should be considered in patients with aCML due to favorable long-term outcomes when performed in CP(53).

MONITORING

The treatment with TKIs requires accurate methods to monitor the drug’s response. The cytogenetic and molecular monitoring can help selecting the most convenient TKI drug and optimize the treatment, both are recommended for a reliable result. The efficacy of TKIs is monitored by complete blood count (CBC) to determine hematologic response (HR). The presence of the Ph chromosome should be monitored by conventional cytogenetic analysis to obtain complete cytogenetic response (CCR) or molecular response (MR) by analysis of transcription levels of BCR-ABL1 by RQ-PCR(47). The cytogenetics control can be used for frequent monitoring a patient with possible change in therapy in case of treatment failure. FISH can replace chromosomal analysis only if bone marrow cells are not obtained and/or for the definition of complete cytogenetic remission (CCyR). Molecular monitoring is performed by RQ-PCR on blood cells every three months until the major molecular response (MMR) is reached, maintaining the investigation every three to six months. Mutational analysis is recommended in cases of progression or failure(47).

According to The European LeukemiaNet (ELN) (2013), the monitoring of CML after the diagnosis follows with chromosomal analysis of bone marrow in 3-6 months and then every six months until CCyR. Tests should be repeated at least every 12 months, only if the molecular response cannot be measured(43).

Monitoring cytogenetic and molecular responses to treatment emerged as a success factor to deal with a long-term disease. The CCyR is defined as the absence of Ph+ cells in at least 20 bone marrow cells analyzed in the metaphases stage. Patients with rapid cytogenetic or molecular remission (before three months) and CCyR have a favorable prognosis; more than 70% of them remain alive after 10 years. Karyotype is crucial in post-remission therapy decision and molecular factors will determine treatment in individuals with normal karyotype. The excessive monitoring can have a high economic cost, but treatment failure may result in acceleration of the disease or death of the patient(48, 52).

TKIs are the first-line treatment for patients with CML in developed countries. HSC transplantation is an alternative to failed TKI treatments or in cases of disease progression. In underdeveloped countries, the treatment and monitoring of patients with CML do not comply with established international guidelines and are not performed properly due to the high cost of medicines, generating great economic impact on the health system(60).

CONCLUSION

Knowledge about CML has advanced considerably in the last decade. By understanding the molecular mechanisms involved in this disease, it was possible to develop new drugs that improve the survival of patients. Therapies that specifically block this neoplasm have obtained promising results and have become a milestone in the treatment of cancer. However, due to genetic instability of leukemic cells, they are susceptible to multiple and heterogeneous mutations. Thus, cytogenetic and molecular diagnostic tests can help the physician to better understand how CML acts in each patient to choose the most appropriate therapy for each one.

The molecular biology and cytogenetic plays an important role in the detection of chromosomal abnormalities associated with cancer, as well as in the characterization of new abnormalities that may trigger the disease, increasing knowledge about the genetic mechanisms involved.
RESUMO

Leucemia mieloide crônica (LMC) é a desordem mieloproliferativa mais comum entre as neoplasias crônicas. A história dessa doença se alia ao desenvolvimento de técnicas de análise citogenética em humanos. Foi o primeiro câncer a ser associado a uma alteração cromossômica recorrente, uma translocação recíproca entre os braços longos do cromossomo 9 e 22 — o cromossomo Philadelphia. Este trabalho é uma revisão atualizada sobre LMC, o qual destaca a importância da análise citogenética no monitoramento contínuo e na orientação terapêutica dessa doença. A pesquisa de artigos científicos foi realizada no banco de dados PubMed, usando os descriptores “leucemia”, “leucemia mieloide crônica”, “tratamento”, “diagnóstico”, “cariótipo” e “citogenética”. Livros e sites especializados também foram incluídos. O monitoramento citogenético e molecular detalhado pode auxiliar na escolha do medicamento mais efetivo para cada paciente, otimizando seu tratamento. A citogenética desempenha um papel fundamental na detecção de anormalidades cromossômicas associadas a malignidades, bem como na caracterização de novas alterações que permitem mais pesquisas e ampliação do conhecimento sobre os aspectos genéticos dessas doenças. O desenvolvimento de novas drogas, através da compreensão dos mecanismos moleculares envolvidos, permitirá uma possível melhora na sobrevivência desses pacientes.

Unitermos: leucemia mielógênica crônica BCR-ABL positiva; leucemia; citogenética; cariótipo; cromossomo Philadelphia; terapêutica.

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