Follow-up of the tumor load in patients with de novo chronic myeloid leukemia and in complete cytogenetic remission treated with imatinib in Colombia

Seguimiento de la carga tumoral en pacientes con leucemia mieloide crónica de novo y en remisión citogenética completa tratados con imatinib en Colombia

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the therapeutic response to Imatinib comes mainly from clinical trials and follow-ups made to patients in developed countries. In order to contrast with those results, we embarked on a mission with the objective to evaluate the hematological, cytogenetic, and molecular response in a group of de novo patients and another with complete cytogenetic response in a developing country such as Colombia.

Methods

Design study: This investigation, descriptive in nature, was carried out in Colombia between January of 2008 and December of 2009 with the participation of 6 hematologic centers, and was authorized by a local ethics committee. The therapeutic conducts adopted during the follow-up period depended exclusively and freely on the treating physician according to his/her experience and on the protocols of each hematologic center. The patients were divided in two groups: de novo and complete cytogenetic remission (CCR). The criterion for inclusion in the first group was the possibility of BCR/ABL fusion proven by PCR in peripheral blood in patients who were clinically suspected to have CML, and for the second, confirmation of the complete cytogenetic remission state in the bone marrow; additionally patients had to be over 18 years old and have written consent of their participation.

The follow-up was done over one year of medical visits with hemogram and molecular assessments every three months and chromosomes every six months: visit 0 (inclusion), visit 1 (90 days), visit 2 (180 days), visit 3 (270 days) and visit 4 (360 days), with some visits made at intermediate periods if the hematologist considered it necessary: the drug was administered by the Health Sponsor Company (EPS in Spanish) to which the patient was affiliated. For the de novo group the Sokal Index was calculated resorting to www.leukemia-net.org at the time of inclusion and the hematological response estimated by each physician according to conventional criterions. The bone marrow samples (3-5 mL) and peripheral blood (10-20 mL) were remitted to room temperature thus guaranteeing their arrival before the 24-hour mark to a central laboratory where the cytogenetic and molecular studies were performed.

Cytogenetic analysis: these analyses were performed on bone marrow samples according to published protocols, at the time of inclusion, 6 and 12 months with a minimal recount of 20 mitosis. Upon arrival, the samples were washed three times with a RPMI 1640 solution and were cultured in 10 mL of the same solution with fetal bovine serum (10%) at 37 oC for 72 hours. The mitosis were obtained immediately upon arrival of the samples and every 24 hours, with the purpose of increasing the probability of obtaining evaluable metaphases. Three milliliters of the cell culture were exposed to 10 uL of colchicine (0.06 ug/mL) for 1 hour. After centrifuging, the supernatant was discarded and the cells were submitted to a hypotonic solution of KCl (0.075 M) for one hour. Finally, the cellular pellet was prefixed and washed five times in a Carnoy fixative (methanol: glacial acetic acid 3:1). The microscope slide with the cellular extension was dried at room temperature for 12 hours to later be dyed in a quinacrine solution (0.05%) for 30 minutes to obtain Q bands.

The mitosis images were seen through an Olympus BX40 microscope adapted with a mercury lamp of 100 W and with the Band-View (Applied Spectral Imaging) software the mitosis were captured and a karyotype was constructed. The cytogenetic response was defined according to the percentage of Ph+ cells: complete (0% Ph+), partial (1% -35% Ph+), minor (66%-95% Ph+) and no response (>95% Ph+).

Real-time polymerase chain reaction: The quantification of the BCR-ABL transcription was carried out with a real time PCR (RT-PCR) using TaqMan method over a Light Cycler 480 platform from Roche. The expression of the beta-glucuronidase (GUS) gene was taken as reference for the normalization of the BCR-ABL p210 transcription expression. The oligonucleotide ENF501 (sense), ENR561 (antisense) and the TaqMan ENP541 probe were used to detect the amplification BCR-ABL. As part of our standardization, the mRNA of 30 cases of CML Ph+ de novo was processed in duplicate with RQ-PCR to obtain a median of the [BCR-ABL]X100/[GUS] rate that was taken as a baseline to transform the percentages obtained with a ten-fold logarithmic base. In our case, the median coincided in 100% so that 10% corresponded to a reduction of 1log, 1% of 2log and 0.1% of 3log. Positive and negative controls were included in all of the reactions and the quality of the mRNA and the efficiency of the reverse transcript were judged by the cycle thresholds (Ct) of each sample, so that a Ct higher than 24 was considered inadequate for the analysis for degradation of the mRNA; in this case, the procedure was repeated from the point of extraction of the mRNA. The sensibility was tested using serial dilutions (10^1 a 10^6) of an mRNA (1-5 ug/ul) with Ct < 20. These same curves for BCR-ABL and GUS were used as a reference to calculate the relative concentration of the samples with the software that came with the Light Cycler 480.

Statistical Analysis: For the descriptive component, percentages were used in the case of discrete and median variables along with their corresponding statistical dispersion (standard deviation) for the case of the continuous variables. In order to illustrate and analyze the behavior of the laboratory variables throughout the follow-up, two-way charts were used. The relation between the quantitative measures of the tumor load in peripheral blood and bone marrow was done with Pearson product-moment correlation coefficient. All of the statistical analysis procedures were done with a Stata 5® program. The percentages on each table correspond to the rate of patients that were evaluated at that moment.

Results

Patients: Twenty-five patients were included in the de novo group and 27 in the CCR group, for a total of 52. At the end of the follow-up, 18 patients remained assessable in the de novo group and 19 in the CCR. The losses of the first group were due to three disease-related deaths, one follow-up loss, two to voluntary withdrawals from the study and one to change to another drug; in the second group, one death after an angioedema not related to the illness, three due to therapy failure, two follow-up losses and two voluntary withdrawals. The median dose of imatinib was 400 mg/day, un patient went up to 600 mg and six to 800 mg; when the study was concluded, 4 patients switched to a second generation tyrosine kinase inhibitor (3 dasatinib and 1 nilotinib). After 12 months, 50% of the patients from the de novo group had treatment failure while in the CCR group 11% relapsed. In Table 1, patient’s overall characteristics are shown.
Hematologic response: The novo group responded quickly to the imatinib with complete responses after 3 months of 79.1%, which increased to 84.2% by the end of the study. In the CCR group after 9 months, 13.6% had lost the hematologic response, although at 12 months all the patients that were evaluated remain in CHR. (Figure 1. A and B) shows the evolution of the white blood cell and platelets count in the novo group.

Sokal risk: In the de novo group, 23.8% remained with low risk, 47.6% remained in medium and 28.6% in high. Table 2 shows the cytogenetic and molecular hematologic response rate according to the Sokal risk in the de novo group at 12 months.

Cytogenetic response: Following the 2010 European LeukemiaNet (ELN) criterion, at 6 months 55.5% of the de novo group responded optimally (complete cytogenetic remission plus partial cytogenetic remission), 11% sub-optimal (>35% Ph+) and 33.4% failed (>95% Ph+). At 12 months, 50% reached an optimal response (complete cytogenetic remission), 5.5% suboptimal (less than complete cytogenetic remission) and 44.5% failed (>35% Ph+) (table 3). One patient in this group showed clonal evolution with double Ph+ chromosome. In the CCR group, 5 patients (25%) had lost the response after 6 months, 2 had follow-up loss, 2 remained in relapse and 1 recovered complete cytogenetic response at the 12 month control. Therefore, by the end of the follow-up, 89.4% maintained the complete cytogenetic remission state while 10.6% had relapsed (table 3).

Molecular response: In the de novo group a decrement in the transcript BCR-ABL levels during the year of treatment was observed, reaching complete molecular responses (undetectable transcripts) in 5.6% and 11.8% in the bone marrow at 6 and 12 months respectively. These percentages were co-related with the molecular responses obtained in peripheral blood (figure 1. C and D). Meanwhile, 53% never decreased their molecular tumor load under 1 logarithm (10%), while 35.2% remained between -1 log (1%) and -3 log (0.1%) (Table 4).

The complete molecular response in the CCR group was consolidated from 15.4% at inclusion to 50% at 12 months. Three cases (11.5%) showed expressions of the BCR/ABL transcript above 10% (-1 log) at the time of the inclusion, one fell out of follow-up, another exhibited a cytogenetic relapse at 6 months and maintained it until 12 months, and the last held the complete cytogenetic response with decrease of tumor load (table 4). The correlation between the quantitative measurements of the tumor load in peripheral blood and bone marrow was significant in the respective visits (r=0.72 inclusion visit, r=0.85 visit 2, r=0.66 visit 4) (figure 1. C and D).

Discussion
This investigation evaluated the hematological, cytogenetic, and molecular responses in patients with chronic myelogenous leukemia (CML) treated with Imatinib in the context of a developing country where socioeconomic conditions could influence the outcome of biological responses. Thanks to tyrosine kinase inhibitors, which efficiently inhibit the Ph+ clone, the chromosomal and molecular analysis becomes the cornerstone for the monitoring of the tumor load. The biological responses have been synthesized in international guidelines such as ELN which make clinical decision-making much easier.7 The therapeutic goal according to these guidelines is to reach optimal responses at 3, 6, 12 and 18 months, until complete cytogenetic remissions and major molecular or undetectable transcripts ("complete" remission) can be achieved and sustained.

**Table 1. Overall characteristics of the patients at the time of inclusion**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Novo</th>
<th>CCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>14(56%)</td>
<td>20(74%)</td>
</tr>
<tr>
<td>White blood cells (n)</td>
<td>&gt;10,000</td>
<td>19,863%</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>&gt;12</td>
<td>7(31.8%)</td>
</tr>
<tr>
<td>Cytogenetic response in the novo and CCR groups</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

**Table 2. Responses of the de novo group at 12 months according Sokal risk, *Number (n) of patients assessable for the characteristics.**

<table>
<thead>
<tr>
<th>Sokal</th>
<th>CHR % (n)</th>
<th>CCR % (n)</th>
<th>QPCR&gt;10 % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIGH</td>
<td>23 (3)</td>
<td>11.2 (1)</td>
<td>37.5 (3)</td>
</tr>
<tr>
<td>MEDIUM</td>
<td>31 (4)</td>
<td>44.4 (4)</td>
<td>37.5 (3)</td>
</tr>
<tr>
<td>LOW</td>
<td>46 (6)</td>
<td>44.4 (4)</td>
<td>25 (2)</td>
</tr>
<tr>
<td>TOTAL*</td>
<td>100 (13)</td>
<td>100 (9)</td>
<td>100 (8)</td>
</tr>
</tbody>
</table>

**Table 3. Cyto genetic response in the novo and CCR groups, * Number of patients assessable for the characteristics.**
sustained over time. It is evident that not every optimal hematological response is accompanied by an optimal cytogenetic response and that complete cytogenetic remission is associated with heterogeneity in molecular response, but this correspondence is 100% in the opposite direction. After a new diagnosis, the first awaited response is the hematological, which in our group was 84.2% at 12 months, lower that those reported in other studies (>96%)\(^1,8,9\). The Sokal Index has upheld its predictive relevance regarding complete cytogenetic response: 89% in low, 82% in intermediate and 69% in high, but it loses its significance in the state of complete cytogenetic response\(^1\). As other authors have observed, our patients with low Sokal Index responded more positively to the hematological, cytogenetic, and molecular criteria, thus reaffirming the importance of defining it at the time of a new diagnosis.

The optimal cytogenetic response at 6 and 12 months was of 55.5% and 50% respectively, inferior to that obtained in clinical trials, which vary from 62% to 69%\(^1,10\); however, our percentages are higher than those obtained by researchers in other developing countries which report optimal cytogenetic responses for the same periods of between 30% and 49% (MCgR)\(^1,11,12\). Fifty percent of the novo patients met with the primary refractory criteria for Imatinib due to failed treatments at 6 and 12 months, despite having received the standard dosages or dosages scaled to Imatinib. The probability of obtaining an optimal cytogenetic response from these patients at 18 months is very low. Although not all causes of primary resistance have been established, these include lack of adherence to the treatment, low bioavailability of the drug, pharmacodynamic factors and mutations in the tyrosine kinase region or a combination of the above\(^13,14\). In some essays it has been established that the presences of previous mutations or those occurring within the first months of treatment influence the outcome of treatment response and are associated to a greater probability of progression\(^15,16\). No biological resistance factors were explored in this group of patients but in the future it would be important for all of us to include such analysis as they would allow clinicians to make better decisions regarding the move towards second generation tyrosine kinase. The quantitative molecular evaluations of the BCR-ABL transcripts in real time with PCR are a key part of the CML follow-up, but it requires a highly sensitive, specific, and reproducible test. Our molecular test corresponded well with the cytogenetic response, indicating that it can be used to monitor CML, although it could be improved in regards to some international standards. On the other hand, consensus only exists regarding the intensity of the molecular response at 18 months, when it is considered optimal to reach a decrease equal or below 3 logarithms (RMM) or a suboptimal response with a warning when this response is lost or the levels of the transcript elevate at some point during the treatment\(^17\).

We implemented a previous local standard for the molecular monitoring establishing a baseline obtained from the Ph+ of 30 de novo patients, which served as a reference in order to transform the percentage of the relation [BCR-ABL]X100/[GUS] of each patient into the respective logarithm\(^18,19\). According to this reference, 100% of our novo patients showed expressions greater than 10% (>1 log) at the time of diagnosis, which dropped 53% (in the bone marrow) by the end of the study and were therefore considered primary resistance patients. The 10% limit (>1 log) corresponding to the non-hematological response and non-cytogenetic remission states, with low probability of improving their response further on with Imatinib. Meanwhile, 11.8% of the novo patients reached undetectable BCR-ABL transcripts at 12 months, which constitutes a group of low risk of progression. As has been obser-
This investigation intended to evaluate the tumor load in a series of patients undergoing treatment with Imatinib without interfering with the therapeutic decisions. The important limitation of this study derive from this observational design, such as being unable to control the variables that influence in the responses like the adherence to treatment, undefined criteria used to escalate the dosage of Imatinib, among other things. Also, the small and restricted size of the group and loss of patients limited our use of statistics in order to further inquire about significant differences between categories. In conclusion, the investigation showed that the optimal cytogenetic responses expected in the novo group and the losses in hematological and cytogenetic responses in the CCR group are not similar to those obtained in clinical trials and reported in developed countries. It is important to know whether similar findings occur in other developing countries and we must call attention to search social, biological and clinical-management reasons that may hinder better outcomes. One measure to be taken would be to adhere to international response criteria with greater rigor and we hope the approval of second-generation tyrosine kinase inhibitors as a first line treatment may improve response rates in our medium.

Conflict of interests:
None of authors have conflicts of interest related of this study.

Acknowledgments:
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References:
22. Marin D, Milojkovic D, Olavarria E, Khorashad JS, de Lavallade H, Reid AG. European LeukemiaNet criteria for failure or suboptimal response reliably identify patients with CML in early chronic phase treated with Imatinib whose eventual outcome is poor. Blood 2008; 112: 4437–44.

Table 4. Molecular response in blood and bone marrow in the novo and CCR groups

<table>
<thead>
<tr>
<th>RESPONSE</th>
<th>0 MONTHS</th>
<th>3 MONTHS</th>
<th>6 MONTHS</th>
<th>9 MONTHS</th>
<th>12 MONTHS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Novo (%)</td>
<td>Novo (%)</td>
<td>Novo (%)</td>
<td>Novo (%)</td>
<td>Novo (%)</td>
</tr>
<tr>
<td></td>
<td>Ccr (%)</td>
<td>Ccr (%)</td>
<td>Ccr (%)</td>
<td>Ccr (%)</td>
<td>Ccr (%)</td>
</tr>
<tr>
<td></td>
<td>SP (%)</td>
<td>SP (%)</td>
<td>SP (%)</td>
<td>SP (%)</td>
<td>SP (%)</td>
</tr>
<tr>
<td>0</td>
<td>0 (15.4)</td>
<td>0 (9.1)</td>
<td>0 (2.9)</td>
<td>0 (5.4)</td>
<td>0 (20)</td>
</tr>
<tr>
<td>0.1-0.99</td>
<td>1 (13.6)</td>
<td>1 (8.7)</td>
<td>1 (16.7)</td>
<td>1 (18.3)</td>
<td>1 (19)</td>
</tr>
<tr>
<td>1-9.9</td>
<td>2 (58.8)</td>
<td>2 (8.7)</td>
<td>2 (16)</td>
<td>2 (23.8)</td>
<td>2 (19)</td>
</tr>
<tr>
<td>&gt;10</td>
<td>1 (110)</td>
<td>1 (110)</td>
<td>1 (110)</td>
<td>1 (110)</td>
<td>1 (11)</td>
</tr>
</tbody>
</table>

This table shows the molecular response in blood and bone marrow in the novo and CCR groups.