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Imatinib mesylate therapy in chronic myeloid leukemia patients in stable complete cytogenic response after interferon-alpha results in a very high complete molecular response rate

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Abstract

To determine the impact on minimal residual disease by switching to imatinib chronic phase chronic myeloid leukaemia (CP-CML) patients responsive to interferon-alpha (IFNα), in stable complete cytogenetic response (CCR) but with persistent PCR positivity.

Twenty-six Philadelphia positive (Ph+) CML patients in stable CCR after IFNα but persistently positive at PCR analysis during this treatment, were given imatinib mesylate at standard dose.

At enrolment into the study, median IFN treatment and CCR duration were 88 months (range 15–202) and 73 months (range 10–148), respectively. Imatinib treatment resulted in a progressive and consistent decline of the residual disease as measured by quantitative PCR (RQ-PCR) in all but one of the 26 patients; at the end of follow-up, after a median of 32 months (range 21–49) of treatment, a major molecular response (BCR/ABL levels <0.1) was reached in 20 patients (77%), and BCR/ABL transcripts were undetectable in 13 (50%). The achievement of molecular response was significantly correlated with post-IFN baseline transcript level (mean 1.194 for patients achieving complete molecular response versus 18.97 for those who did not; $p < 0.001$), but not with other clinical/biological disease characteristics.

These results indicate that patients induced into CCR by IFN treatment represent a subset with very favourable prognosis, which can significantly improve molecular response with imatinib and further support investigative treatment schedules combining these two drugs.

Keywords: Chronic myeloid leukemia; Interferon alpha; Imatinib; Minimal residual disease

1. Introduction

Chronic myeloid leukemia (CML) is a malignant hematopoietic disease whose molecular hallmark is the BCR/ABL gene rearrangement originating from a t(9;22) translocation. The BCR/ABL fusion product encodes for a deregulated tyrosine kinase (TK), which has a central role in the pathogenesis of the disease [1]. Until recently, interferon-α (IFN) was considered the gold standard for drug therapy of CML, as it yielded complete cytogenetic response (CCR) in 10–25% of patients with significant survival prolongation, particularly in low risk patients usually obtaining a higher response rate [2,3]. However, even in best responding patients, the disease still remained detectable at a molecular level, and the majority of patients eventually relapsed [4–7]. Only consistently negative RT-PCR patients

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appeared to remain 100% in CCR at 10 years follow-up [7].

The introduction of imatinib mesylate (STI571, Gleevec), a selective inhibitor of the BCR/ABL TK, has revolutionized the disease management, as it induces CCR in 50–90% of chronic phase (CP) CML patients, including those resistant or refractory to IFNα [8,9]. Furthermore, molecular monitoring using quantitative PCR (RQ-PCR) has shown a consistent reduction of the transcript level in a high proportion of responder patients, and the degree of the molecular response strongly correlates with the probability of progression-free survival. A reduction of the BCR/ABL transcript level ≥3 log at 12 or 18 months predicts almost 100% long-term remission [8–12]. However, residual disease still remains detectable using PCR standard procedures in the majority of patients, and development of imatinib resistance is the main cause of therapy failure [11–17].

In this study, we administered standard dose imatinib to 26 CML patients in late CP, who were in stable CCR induced by IFN, but had persistent residual disease by molecular analysis. We monitored the level of BCR/ABL fusion transcript by RQ-PCR to assess the impact on residual disease of crossover from IFN to imatinib in this subset of IFN-responding patients and the possible correlations between response to imatinib and clinical-biological characteristics of patients at diagnosis and during follow-up.

2. Materials and methods

2.1. Patients and study design

Twenty-six Ph+ CP-CML patients who had been diagnosed in our institutions between December 1985 and March 2000 entered this study. Inclusion criteria were: (a) having received an IFNα based treatment, (b) being in stable CCR defined as the absence of Ph+ mitoses in at least two consecutive analyses (6 months apart), and (c) being persistently positive at qualitative PCR analysis for the BCR/ABL transcript.

At presentation all patients were previously untreated and in first CP according to the standard criteria [18]. All patients had been receiving IFN from diagnosis at the maximum tolerated dose (up to 5 MU/m^2/day); following CCR, the initial IFN scheduled dosage was adjusted to maintain WBC count between 1.5 and $4 \times 10^9\,L^{-1}$. From the time of imatinib treatment, quantitative PCR analysis of residual disease was regularly assessed. A reduction in the dose of imatinib because of non-hematologic or hematologic toxicities was allowed according to common toxicity criteria [18]. Complete blood counts and serum chemistry evaluations were performed before starting imatinib, weekly during the first 6 weeks, every 2 weeks for the next 6 weeks, and every 6 months thereafter.

2.2. Cytogenetics

Cytogenetic analyses were performed on bone marrow (BM) aspirates at diagnosis, every 6 months during IFN treatment, before starting imatinib (baseline), and after 3, 6, and 12 months of therapy and thereafter every 6 months, according to standard methods. Only results obtained from at least 20 metaphases were considered as evaluable. Cytogenetic response was categorized according to standard criteria [2]: CCR was defined as the presence of 100% Ph- metaphases.

2.3. FISH analysis

FISH analysis was performed on unseparated nucleated cells using differently labeled BCR and ABL probes (LSI BCR/ABL ES Dual Color Translocation Probe, Abbott Molecular Diagnostics) with a minimum of 150 cells in interphase being scored for each sample. Normal cells display two red signals (ABL gene) and two green signals (BCR gene), while the FISH pattern of Ph positive cells consists of one red, one green and two yellow signals. The cut-off limit for BCR/ABL positive analysis was set at 0.2%, i.e. the mean + 2 S.D. of positive FISH pattern detected by scoring 1000 nuclei of bone marrow cells from patients affected by other hemopoietic diseases.

2.4. Quantitative evaluation of minimal residual disease

2.4.1. Organization, sampling schedule and storage of samples

Quantitative assays of minimal residual disease (MRD) were centrally performed in Naples at the CEINGE-Biotecnologie Avanzate [19]. BM samples for MRD analysis were collected from all patients enrolled into the study prior to imatinib treatment (baseline), after 3, 6, 12 and 18 months; a further assay of MRD was also carried out at the latest follow-up.

2.4.2. Cell separation and RNA extraction

Leukocyte pellets were isolated from BM aspirates by lysis of red blood cells, and re-suspended in aliquots of 5×10^6 in 600 μL of 4 M guanidium isothiocyanate solution (GITC). Total RNA was extracted using ion exchange chromatography on minicolumn (GeneElute, Total RNA Purification kit, Sigma, St. Louis, MO, USA), according to the manufacturer directions.

2.4.3. Real time quantitative RT-PCR assay of minimal residual disease

MRD was detected during follow-up by a recently standardized RQ-PCR method [20]. The method independently measures in each sample by real time PCR, the copy number of both mRNA encoding for the P210 BCR/ABL protein and for Abelson (ABL); the latter was used as a control gene to verify sample-to-sample RNA quality variations. In this study, for each amplification run, both a BCR/ABL and ABL

standard curve were independently generated by assaying, in parallel with the samples, 1:10 serial dilutions (from 10^6 to 10^2 copies, each in triplicate) of plasmid DNA calibrators containing the target sequences diluted in a solution of *E. coli* RNA (20 ng/μL). The copy number (CN) of BCR/ABL and ABL transcript are derived by the interpolation of the cycle threshold (C_t—the number of PCR cycles necessary to detect a signal above the threshold) values to the appropriate standard curve; the result for each sample was expressed as ratio of BCR/ABL mRNA copies to ABL mRNA × 100 (normalized copy number—NCN).

2.4.4. Real Time quantitative PCR reaction conditions

The reaction conditions were the same for both BCR/ABL and ABL mRNA RQ-PCR. Briefly, 1 μg of total RNA extracted from the patient samples was pre-warmed for 10 min at 70 °C and incubated for 10 min at 25 °C; the RNA solution was then incubated for 42 min at 45 °C in a 20 μL reaction mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5.5 mM $MgCl_2$, 1 mM of each deoxyribonucleotide, 20 U of RNAsin (Pharmacia, Upsala, Sweeden), 25 mM random examers (Pharmacia), 10 mM of DTT (Pharmacia), and 100 U of MoMLV reverse transcriptase (BRL, Bethesda, MD). PCR amplification of p210- and ABL-encoding cDNAs were separately carried out in a reaction mixture consisting of 1 × Master Mix (Applied BioSystem, Foster City, CA USA), 300 nM of the appropriate primer pair and 200 nM of the appropriate probe in a final volume of 25 μL using the following time/temperature profile: 95 °C, 15 s, and 60 °C, 1 min, for 50 cycles. All amplification reactions were carried out in triplicate. Primers and probe sequences for RQ-PCR of BCR/ABL and ABL were designed, tested and standardized within the EU concerted action (Table 1). Plasmid dilution used to generate the standard curves of the assays, were purchased by IPSOGEN Inc. (Marseille, France).

Sample results and all analytical series underwent to a rigorous check, as follow: (i) RNA samples that repeatedly gave ABL Ct values higher than 28.7 were operationally considered degraded and eliminated from further evaluation to ensure a sensitivity of at least 4 logs in all samples assayed; (ii) in the case of BCR/ABL C_t value higher than the intercept value of the relative standard curve of the run (C_t value corresponding to one copy), the samples were considered negative (MRD below the detection limit of the technique); (iii) in the case that the slope of a standard curve was not comprised within the mean ±2 standard deviations, all the samples of the analytical series were re-assayed.

Table 2
Patient features

Number of patients	26
Age	
Mean (years)	40
Range	21–64
Sokal Risk	
Low	18
Intermediate	8
High	–
Follow-up (months)	
Median	122
Range	49–236
IFN-α treatment	
Duration (months)	
Median	88
Range	15–202
Weekly dose (MU)	
Median	26
Imatinib treatment	
Duration (months)	
Median	32
Range	21–49
Daily dose (mg)	
Median	400

2.5. Statistics

Statistical analysis was performed using SPSS version 6.0 software; Wilcoxon–Mann–Whitney test was performed for comparison of non-parametric series and Fisher's exact test was used to compare categories. Values of $p < 0.05$ were considered of statistical significance.

3. Results

3.1. Pre-Imatinib features of patients

Clinical features of the 26 patients enrolled into the study are summarized in Table 2.

At diagnosis median age was 40 years (range 21–64), and baseline Sokal risk score was low in 18 patients and intermediate in the remaining 8, whereas no patient was in high risk

Table 1
Sequences of primers and probes used for the assay of BCR/ABL and ABL mRNA levels

#	Sequence (5′ → 3′)	Description
ENF501	TCCGCTGACCATCAACAAGGA	Sense—BCR exon 13
ENP541	Fam-CCCTTCAGCGGCCAGTAGCATCTGA-Tamra	Probe—ABL exon 2
ENR561	CACTCAGACCCTGAGGCTCAA	Antisense—uABL exon 2
ENF1302	GAGTATGCCTGCCGTGTG	Sense—ABL exon 2
ENPr1342	Fam-CCTCCATGATGCTGCTTACATGTCTC-Tamra	Probe—ABL exon 3
ENR1362	AATCCAAATGCGGCATCT	Antisense—ABL exon 4

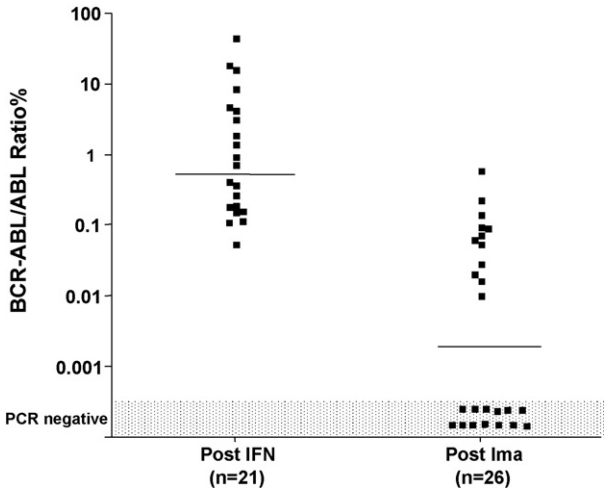

Fig. 1. MRD levels at baseline and at end of follow-up. MRD levels assessed by RQ-PCR in patients at IFN treatment discontinuation before imatinib therapy and at the end of the follow-up. Dotted lines indicate median values of the two groups of MRD values.

category. Overall, median weekly dose of IFN received from the patients was 26 MU, the median duration of IFN treatment being 88 months (range 15–202). All the 26 patients had sustained CCR from a median of 73 months (range 10–148) at the start of imatinib. Total follow-up from diagnosis of the patients was 122 months (range 49–236). During the course of IFN treatment, qualitative RT-PCR analysis tested always positive for all the patients. At the end of IFN treatment, median BCR/ABL transcripts level in BM samples of patients, assayed by RQ-PCR analysis, was 0.89 (range 0.06–100.04), and was considered as study baseline level of MRD (see Fig. 1). In three patients, RQ-PCR showed a level of MRD higher than 10, despite the confirmed status of CCR. Therefore, FISH analysis was performed on interphase marrow cells of patients given the possible presence, in the marrow of the three patients, of Ph positive cells still inhibited by IFN in their capability to proliferate in vitro. Consistently with the RQ-PCR data, all these patients had up to 8% of analyzed nuclei positive for the BCR/ABL translocation, thus suggesting the possibility of an impending disease relapse (Table 3). None of the other patients had BCR/ABL positive cells detectable by interphase FISH.

Table 3
FISH analysis of the three patients with high MRD level at the treatment switch to Imatinib

Patient	IFN treatment duration (months)	MRD level	Interphase FISH	
			Analyzed nuclei	BCR/ABL+ve (%)
#1	202	52.84	291	8
#2	66	100.04	190	7
#3	96	20.77	290	2

3.2. Imatinib treatment

Imatinib treatment was well tolerated by all 26 patients; no patient needed dose reduction or drug discontinuation for hematological or extra hematological side effects. At the present time, after a median of 37 months (range 26–54) of clinical observation, all patients are still on imatinib treatment at the full 400 mg/die dose. No patient showed loss of either hematological or cytogenetic complete response. Overall, total follow-up from diagnosis of the 26 patients is 122 months (range 49–236).

Imatinib treatment induced in all but one patient a significant decrease of MRD level: median BCR/ABL ratio reached <0.01, a level more than two-log lower with respect to the baseline post-IFN value (Fig. 1). Noteworthy, 20 patients (77%) had PCR results below 0.1%, with 13 of them (50%) being in complete response (BCR/ABL transcript not detectable by PCR) (Table 4). The levels of MRD at the end of follow-up did not correlate with overall disease length, IFN treatment duration and the Sokal risk at presentation (data not shown). Conversely, the levels of MRD reached after IFN (baseline level in this study) of the 13 patients who obtained complete molecular response under imatinib were significantly lower than those of the remaining patients (1.194 versus 18.97, $p < 0.001$). Sensitivity of PCR analysis was assessed as previously published in single patient samples [19] to avoid any influence on results by sample degradation. Mean ABL copy number of the analyzed samples was 8500 (range 1300–48,000) with a calculated sensitivity of the assays never lower than 10^{-4}, and in the case of samples who gave negative PCR results the mean ABL copy number was 7200 (range 1300–21,100). Overall, MRD monitoring (Fig. 2) showed a progressive decline of median values over the various time points, thus indicating a continuous reduction of the leukemic burden. Remarkably, the response to imatinib treatment was very rapid in a significant proportion of patients; MRD level was evaluable in 18 samples after the first 3 months of imatinib treatment, and in seven of these BCR/ABL transcripts were no longer detectable (PCR negative), while in other two the level was below 0.1 (major molecular response) (Fig. 2). In a single patient, MRD level showed almost one log increase at the last follow-up. This patient had, at presentation, an intermediate Sokal risk of progression, and, at start of imatinib treatment, more than 15

Table 4
Minimal residual level by RQ-PCR of BCR/ABL in the 26 patients

	Baseline	Last follow-up
Mean[a]	10.51	0.09
Median[a]	0.89	0.01
Range[a]	0.06–100.04	0–1.02
MRD <0.1 (no.)	1	20[b]
PCR negative (no.)	–	13[c]

[a] Value expressed as BCR/ABL-ABL ratio%.
[b] Including the 13 PCR negative patients.
[c] Baseline MRD level of these patients was 1.194 vs. 18.97 ($p < 0.001$) of the remaining patients.

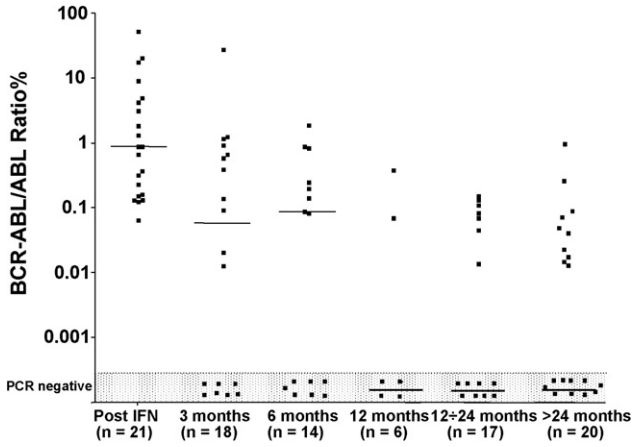

Fig. 2. Progressive effect of imatinib on MRD level. Representation of the progressive decline of MRD levels observed in CML patients under imatinib treatment. Dotted lines indicate median values of MRD at single time points.

years of IFN therapy with an overall follow-up duration of 220 months.

4. Discussion

In the present study, we analysed the effect of switching to imatinib 26 CML patients with long term cytogenetic but not molecular response to IFN. The main reason for this therapeutical decision was to evaluate whether a stable cytogenetic response to IFN could be improved at the molecular level through the possible complementary actions of the two drugs.

We found that 77% of our patients treated with a standard dose of imatinib reached BCR/ABL levels <0.01 over a median follow-up period of 32 months. These data were obtained from an unusual subset of selected patients; therefore, they cannot be extrapolated to the general CML patient population. However, some considerations may be done. Using IFN, 10–25% of CP-CML patients obtain CCR, but only a minority of them achieve and maintain very low levels of residual disease and do not experience disease relapse or progression [4–7,21,22]. When imatinib is given at 400–800 mg/day, CCR is obtained in 50 to >90% of patients with new diagnosis or late CP-CML, including those pre-treated with IFN [9,23,24]. Furthermore, molecular monitoring by quantitative RQ-PCR, has shown consistent reduction of the transcript level in a high proportion of responder patients, and the degree of the molecular response strongly correlates with the probability of long-term progression-free survival [12,17,24]. However, transcript undetectability only reached a limited number of patients. The dynamics of the molecular response has been analysed in large series of patients in CCR after imatinib, the percentage of cases with undetectable transcripts ranging from 4 to 8% to near 40% of the cases, depending on disease status (late or early CP), prior IFN treatment or not, imatinib dosage (400 or 800 mg/day) [9,23]. Therefore, the heterogeneity of previously investigated cases precludes direct comparison with the data obtained from the present study.

In our series of selected patients in minimal disease status, the molecular response was very rapid and residual disease undetectability was achieved in a high proportion of patients. Furthermore, transcript reduction in almost all our patients appeared to be sustained, and progressively increasing over time, thus indicating ongoing disease depletion, as previously observed [9,12,13]. Also the three patients who had a high MRD level following IFN treatment showed a rapid decline of the BCR/ABL transcript levels as assessed by RQ-PCR. This subset of patients with a high level of BCR/ABL transcript was already indicated in the IFN era as candidate for an imminent relapse [4]; noteworthy, these three subjects showed a small percentage (2–8%) of BCR/ABL positive nuclei at FISH analysis and they were likely going into relapse.

Despite being in late disease, our patients obtained improved molecular response on imatinib. This apparently contrasts with the observations that better results are usually reached in earlier disease due to the enhanced probability of developing imatinib-resistant mutations over time [9,16,25]. We could thus infer that the significant reduction of the BCR/ABL positive cells induced by IFN made the development of resistance less likely to occur in our patients. Alternatively, some biological factors may exist in this subset of patients with particularly good prognosis, capable of preventing clonal evolution. We only observed a rough correlation between the baseline (post-IFN) level of BCR/ABL transcripts and the degree of molecular response under imatinib, while we did not find, in our series of patients, a correlation between molecular response to imatinib and disease duration, length and weekly dose of IFN, and Sokal score. It should be underlined that no high Sokal risk patient was included in our study group, may be as a consequence of the unsatisfactory response to IFN usually seen in this subset of CML patients. The prognostic impact of high Sokal risk was also confirmed in imatinib era by large cooperative clinical trials: the CCR rate in this subgroup of patients never exceeds 70% even under high dose imatinib treatment [17,23]. Finally, the mechanisms by which imatinib improved molecular status in our IFN-responsive patients remain to be elucidated. It is well known that these two drugs act through different molecular mechanisms, and it is thus expected that their effects may be mutually potentiated [26]. The complex action of IFN, which includes direct antineoplastic effect and immunomodulatory activity, is not fully understood [26]. However, it has been suggested that it induces control, rather than eradication, of the disease [27]. In contrast, imatinib and the new generation TK inhibitors act directly on the oncogenic protein of the leukemic clone; however even imatinib may fail to completely eliminate primitive quiescent Ph+ progenitor cells [27]. Thus, it is possible that IFN and imatinib exerted a complementary effect in our patients, while showing no cross drug resistance.

Much has been discussed about curtailing treatment in patients with persistent CCR after IFN, given that very low number of Ph+ cells does not necessarily imply relapse, and that patients with molecularly negative disease do not seem to experience relapse [5,6,7]. This issue applies now also to patients in CCR after imatinib, especially to those achieving persistently undetectable levels of residual disease, in whom divergent outcome have been observed after imatinib discontinuation [28]. Furthermore, as a high number of patients in CCR on imatinib still remain molecularly positive and at potential risk of relapse, strategies aiming at increasing PCR negativity need to be explored, which include combining or adding synergistic drugs to imatinib [29].

The concurrent use of imatinib and IFN has been recently studied in multicenter trials but results have demonstrated additive cytotoxic effects, limiting the possibility to apply this approach in the clinical practice [26]. Though the use of imatinib is at present the first choice for CML therapy, the complementary actions of the two drugs could be investigated in sequential or low-dose combination, also in patients reaching stable CCR on imatinib but still having detectable molecular disease.

Longer follow-up and strict monitoring of these patients, with improving technologies over time is required to determine to what extent they will become molecularly negative and whether patients with undetectable transcripts are indeed cured of their disease.

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