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Assessment of the response to imatinib in chronic myeloid leukemia patients – comparison between the FISH, multiplex and RT-PCR methods

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Abstract: *Objective*: The objective of this study was to evaluate the kinetics of molecular response in chronic myeloid leukemia (CML) patients treated with imatinib and to compare between the fluorescent *in situ* hybridization (FISH), multiplex and real-time quantitative RT-PCR (RQ-PCR) methods with this respect. *Methods*: Molecular follow-up was carried out on 24 CML patients treated with imatinib. FISH analysis was performed according to the standard protocol. For RT-PCR the multiplex and RQ-PCR methods were used. *Results*: Sixty-three percent and 52% of the patients achieved complete remission according to FISH and multiplex RT-PCR analyses, respectively. Seventy-five percent of the patients achieved remission within the first year of treatment. In 83% of the cases the FISH and RT-PCR results were concordant. RQ-PCR analysis was carried out on 32 of the 41 samples negative by multiplex RT-PCR but only nine were negative. All samples with a BCR–ABL/ABL ratio below 2% were also negative by FISH. There was an excellent correlation between the RQ-PCR and the FISH tests. *Conclusions*: Molecular remission according to FISH and multiplex RT-PCR can be achieved by imatinib within 1 yr of therapy. There is a good correlation between the FISH, multiplex and RQ-PCR results in terms of the kinetics of disappearance of the BCR–ABL transcript and the predictability of each method for the other. Although RQ-PCR is the most sensitive method for molecular follow-up, FISH and multiplex RT-PCR can be used as complementary tools, at least during the early period of treatment.

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The introduction of imatinib mesylate (imatinib, formerly STI 571), a compound that inhibits the tyrosine kinase activity of BCR–ABL, has shown promising results in patients with chronic myeloid leukemia (CML) (1–5). Response is expressed at three levels: hematological, cytogenetic, and molecular. Minimal residual disease (MRD) analysis in patients with CML is of major importance in the assessment of the patient's response to treatment (6–8). The ideal test for the monitoring of MRD should be sensitive, easy to perform, and frequent testing, preferably by using peripheral blood, should be possible. Although qualitative RT-PCR for BCR–ABL is by far the most sensitive assay in the context of MRD analysis, a single positive result cannot predict relapse in an individual patient. In view of the very limited value of qualitative PCR, several groups have developed quantitative PCR assays to estimate the amount of residual disease in positive specimens. Most groups

have initially used competitive PCR strategies. Recently, novel real-time quantitative PCR procedures (RQ-PCR) have been developed that promise to simplify existing protocols (5–8).

We decided to analyze retrospectively our MRD follow-up results of 24 patients treated with imatinib. Our aims were to evaluate retrospectively the rate and kinetics of molecular response to imatinib by using the fluorescent *in situ* hybridization (FISH), multiplex RT-PCR and RQ-PCR methods, to compare between these methods for evaluation of molecular response and to assess the rate of 'true' molecular remission of patients treated with imatinib.

Patients and methods

Patients

Twenty-four CML patients (15 males, nine females) treated with imatinib had molecular follow-up in our institute. Their results were analyzed retrospectively. Mean age at the start of imatinib therapy was 51 (range 23–72) yr. Mean disease duration before imatinib therapy was 3.3 (range 0.1–11.8) yr. Seventeen patients were treated in chronic phase (CP), four patients in accelerated phase (AP) and three patients in blastic crisis (BC). Twelve patients in CP received imatinib for interferon-resistant disease and five patients received it because of intolerance to interferon. Patients received 400 mg imatinib daily for CP CML and 600 mg daily for AP and BC with the option of dose adjustment according to the clinical course. The median follow-up from the initiation of imatinib to the last test obtained was 14.9 (range 1–30) months.

Methods

FISH analysis

The samples for this study were bone marrow (BM) and peripheral blood (PB) mononuclear cells from consenting CML patients treated with imatinib. As previously shown, the sensitivity of PB is similar to that of BM for FISH and RT-PCR analyses in CML patients (9–11). FISH analysis was performed by hybridization with probes specific for the BCR and ABL on interphase nuclei of the patients. The majority of FISH tests were preformed according to the standard FISH protocol developed in our laboratory using the modified BCR–ABL extra signal (es) probe (Vysis, Downers Grove, IL, USA) as previously described by us. The remaining samples were analyzed by the older conventional method. These were samples analyzed before the more sensitive (es) probe was introduced in our lab (12, 13). Because the FISH (es) method has false-positive (FP) rate of 1%, results were considered negative when $<1\%$ of interphase nuclei were positive for BCR–ABL double-labeled probes. Accordingly, we divided our patients into those who did or did not achieve complete remission (CR) according to whether they had more or $<1\%$ of BCR–ABL positive cells.

Multiplex RT-PCR analysis

Total RNA was isolated using the RNAeasy kit (Quiagen, Hilden, Germany) following the manufacturer's instructions. RNA was transcribed into cDNA and multiplex RT-PCR was performed as previously described by Cross *et al.* (14). It was performed to distinguish almost all the known BCR–ABL transcripts. The sensitivity of this test ranged from $1:10^2$ to $1:10^3$ (14). The multiplex assay also amplifies an 808-bp fragment of the normal BCR transcript as an internal control of RNA quality, cDNA preparation and RT-PCR efficiency. Results were considered negative if there was no BCR–ABL band but only if the BCR band was clearly visualized. Patients were divided into those who did or did not achieve CR according to whether they had a negative result as defined.

Real-time quantitative RT-PCR

All RQ-PCR reactions were performed on a 7900HT ABI platform using primers and TaqMan probes kindly provided by Applied Biosystems (Foster city, CA, USA) in conjunction with the TaqMan Universal Master Mix purchased from the same manufacturer.

The threshold was systematically set at 0.1 in order to avoid any particular problem of baseline creeping. The baseline was set up between cycles 3 and 15. The control gene was ABL. Optimum probe and primers were designed using the Primer Express software (Applied Biosystems), the sequences and their positions are as follows: BCR (forward primer) – 5′–3′ position: 1727–1744; sequence 5′–3′: CTG GCC CAA CGA TGG CGA; ABL (reverse primer) – 5′–3′ position: 277–257; sequence 5′–3′: CAC TCA GAC CCT GAG GCT CAA; ABL (TaqMan probe): 5′–3′ position: 230–254; sequence 5′–3′, CCC TTC AGC GGC CAG TAG CAT CTGA. The Taq-man probes were dual labeled with reporter FAM on the 5′-end for BCR–ABL and VIC for ABL and quencher TAMRA on the 3′-end. The quantitative RQ-PCR assays for the quantification of BCR–ABL were designed to detect b2a2 and b3a2 transcripts in a single reaction.

The BCR–ABL and ABL mRNA levels were quantified by subjecting cDNA to 50 cycles of RQ-PCR using the ABI 7900 Sequence Detection System (Applied Biosystems) and TaqMan

Universal Master Mix (dATP, dCTP, dGTP, dTTP: 200 μM, $MgCl_2$ 5.5 mM, AmpliTaq-Gold: 0.025 μM) in accordance with the manufacturers instructions, in a final reaction volume of 25 μl. Reaction conditions and primer selection for optimal amplification of both BCR–ABL and ABL were determined as described in the manufacturer's manual. The level of expression of each gene was measured as copy number off the standard curve.

The number of ABL gene transcripts was quantified to control for the integrity of the RNA and the BCR–ABL copy number normalized with respect to its copy number and expressed as percentage ratio of BCR–ABL/ABL. Results were scored as negative only if BCR–ABL transcripts were not detected and the ABL transcripts were $\geq 1 \times 10^4$. Accordingly patients achieved CR if they had a negative result thus defined.

An ABL assay was performed in duplicate and a BCR–ABL assay performed in triplicate.

The standard curve generated using serial dilutions of linearized plasmid, containing BCR–ABL insert FGRS10. The standards assayed ranged from 10^2 to 10^6 copies per 5 μL. The plasmid used to quantify the endogenous control gene ABL was CGRS1 (Ipsogen, Marseille, France). The standards were limited to 10^3–10^5 in 1 log increments.

Statistical analysis

For categorical variables frequencies, percentage, and distributions were summarized. For continuous variables ranges, medians, mean and standard errors were summarized. The mean between pairs of continuous variables were analyzed by the '*T*-test for paired differences'. The median ranges between pairs of continuous variables were analyzed by the 'Wilcoxon' rank test. The correlation coefficients between pairs of continuous variables were calculated by Pearson correlation. The significance level of each correlation coefficient was analyzed. Significant level for all statistical tests was 0.05.

Results

Response rate

FISH analysis

Twenty-four patients underwent FISH analysis. Ninety-four FISH tests were carried out with a median of three tests per patient (range 1–22). Seventy-nine of the 94 tests were performed by the modified extra signal probe FISH (es) protocol. The median number of cells scanned per sample was 300 (range 100–1000). According to our previous experience, in most cases, a minimum of 300 cells must be counted for reliable analysis. The wide range of scored cells stems from the fact that a small number of cells had to be scored for samples with a high percentage of BCR–ABL fusion and vice versa (12). Fifteen patients (63%), 13 in chronic phase and two in accelerated phase, achieved CR according to FISH analysis. Thirteen patients remained in CR and two patients progressed during the follow-up period.

Table 1. Classification of RQ-PCR results according to BCR–ABL/ABL ratio

BCR–ABL/ABL	Tests *n* (%)
0%	9 (28)
>0% and ≤ 2%	17 (53)
>2%	6 (19)
Total	32 (100)

Multiplex RT-PCR analysis

Sixty-five multiplex RT-PCR tests were carried out on 21 patients with a median of two tests per patient (range 1–24). Eleven patients (52%) achieved PCR negativity. All 11 patients remained PCR-negative.

Real-time quantitative RT-PCR

RQ-PCR analysis was carried out on 32 of the 41 (78%) multiplex-negative samples (Table 1). In 21 of these 32 cases FISH results were obtained concomitantly. Only nine samples, obtained from seven patients (two patients had two negative results each), were negative at a certain time point. The median value of BCR–ABL/ABL ratio of the 23 positive samples was 0.55%, range 0.04–22%. As previous studies showed that all the patients in complete cytogenetic remission had BCR–ABL/ABL ratio $< 2.0\%$, we divided the positive RQ-PCR results into those with a ratio below or above 2% (9, 15). Seventeen of the 32 RQ-PCR tests (53%) had a positive BCR–ABL/ABL ratio below or equal to 2%. Therefore, 81% of all patients with negative multiplex RT-PCR results assessed by us for RQ-PCR had a BCR–ABL/ABL ratio below or equal to 2%. Six of the 32 patients (19%) had a BCR–ABL/ABL ratio above 2%.

Kinetics of molecular response

FISH

Fifteen of the 24 patients assessed by FISH achieved CR. The median time to achieve it was 188 (range 35–526) d. In 75% of the patients CR was achieved within the first year of treatment (Table 2).

Table 2. Kinetics of molecular response

% of patients achieving CR	Time to achieve CR (months)	
	FISH	Multiplex RT-PCR
25	3.8	3.8
50	6.2	6.3
75	6.4	7.3

Multiplex RT-PCR

Eleven of the 21 patients assessed by multiplex RT-PCR achieved PCR negativity. The median time to achieve PCR negativity was 188 (range 46–340) d. In 75% of these patients PCR negativity was achieved within 8 months (Table 2). All 11 patients remained PCR-negative till the end of the study. There was no statistically significant difference between the results of FISH or RT-PCR with respect to the median time that elapsed from the initiation of imatinib treatment to the first negative result ($P = 0.67$).

Correlation between FISH and multiplex RT-PCR results

The mean time between the initiation of treatment and the first documented negative (< 1%) FISH or multiplex RT-PCR result (same patients) was similar – 199 and 184 d, respectively ($P = 0.50$). There was a high Pearson correlation coefficient between the two time periods ($r = 0.93$, $P = 0.0002$) (Table 3, Fig. 1).

We analyzed the results of 45 cases in which both – FISH and multiplex RT-PCR were applied concomitantly to the same patient on the same day (Table 3, Fig. 1).

In 37 of the 45 cases (83%) the FISH and multiplex RT-PCR results were concordant. In 26 of the 45 cases (58%) both FISH and multiplex RT-PCR results were negative and in 11 of the 45 cases (25%) both tests were positive.

In eight of the 45 cases (17%) the FISH and multiplex RT-PCR results were discordant. In six cases (13%) FISH results were negative while multiplex RT-PCR results were positive and in two cases (4%) FISH results were positive while RT-PCR results were negative.

Table 3. Correlation between FISH and multiplex RT-PCR results

	FISH		
	+	–	Total
RT-PCR			
+	11	6	17
–	2	26	28
Total	13	32	45

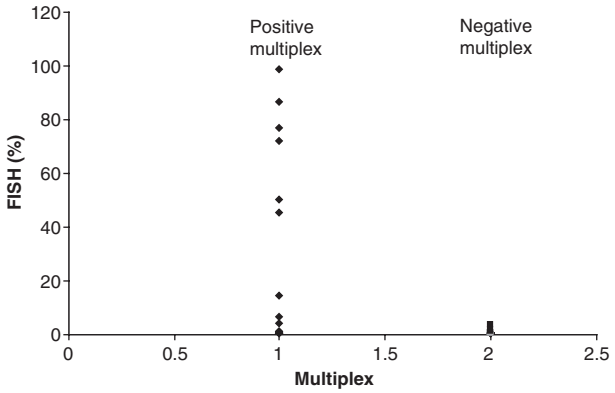

Fig. 1. Comparison of the BCR–ABL levels derived from FISH analyses vs. multiplex analyses.

The sensitivity of the multiplex RT-PCR test relative to the FISH test was 85%. The specificity of the multiplex RT-PCR test relative to the FISH test was 81%. The positive predictive value of multiplex RT-PCR test relative to the FISH test was 65%. The negative predictive value of multiplex RT-PCR test relative to the FISH test was 93%. The false-negative value of the RT-PCR test relative to the FISH test was 15%. The false-positive value of the RT-PCR test relative to the FISH test was 19%.

Correlation between FISH and RQ-PCR results

Twenty-one FISH tests were obtained concomitantly with the 32 RQ-PCR tests. As mentioned before, all 32 RQ-PCR tests were carried out on samples that were multiplex RT-PCR negative. FISH analysis was carried out in five of the nine samples with negative RQ-PCR results. All tests (100%) were FISH negative (< 1%). FISH analysis was carried out on 11 of the 17 samples with RQ-PCR results ≤ 2%. All tests (100%) were FISH negative. FISH analysis was carried out on five of the six samples with RQ-PCR results > 2%. Four of these five tests (80%) were FISH negative and one test (20%) was FISH-positive. Therefore, in 100% of the samples that were multiplex RT-PCR negative and had a RQ-PCR value below or equal to 2% (i.e. low levels, equivalent to complete cytogenetic remission) the FISH results were also negative. The FISH results were also negative in four of the five tests that were multiplex negative but had RQ-PCR values above 2%. Only in one sample, with RQ-PCR value of 22%, the FISH result was positive (Table 4).

There was a strong correlation between the two analyses manifested by a highly significant Pearson correlation coefficient (+0.87; $P = 0.001$) (Table 4). In five samples both FISH and RQ-PCR tests were negative and in one sample FISH test was positive

Table 4. Correlation between FISH and RQ-PCR results

		FISH		
		+	−	Total
RQ-PCR				
	≤ 2%	0	11	11
+	>2%	1	4	5
−		0	5	5
Total		1	20	21

and RQ-PCR ratio was >2%. In additional 11 cases FISH results were negative and RQ-PCR values were equal to or below 2%. Five of the 21 cases were discordant as RQ-PCR values were >2% while FISH results were negative.

Discussion

As the presence of the BCR–ABL gene and its products are considered a surrogate for disease activity its disappearance is regarded as the prerequisite for cure and the ultimate therapeutic goal.

Cytogenetic analysis is still the 'gold standard' in the diagnosis and follow-up of CML. The advantages of cytogenetics are its ability to reveal karyotypic abnormalities in addition to the Philadelphia (PA) chromosome that may arise during disease progression. The main limitations of cytogenetics are its low sensitivity of 1–10% and the need to do the test on bone marrow samples in order to get metaphases (5–9, 16–19). FISH detects BCR–ABL in about 95% of CML cases. A FISH study routinely analyses 200–500 nuclei; thus, quantification generated by FISH is faster, more accurate than cytogenetics, especially when few leukemic cells are present. Also, unlike cytogenetics, which requires dividing metaphase cells, FISH can be performed on interphase nuclei in PB with the same sensitivity as BM. Because of the added accuracy and sensitivity, FISH is being used increasingly as a surrogate to replace cytogenetics in monitoring of CML patients (5, 20–22). Several authors studied whether FISH on PB could be a valid alternative compared with BM karyotyping or FISH. Some found a good correlation, but discrepancies between both methods in individual patients and between studies were found by others (20, 22–25). The proportion of circulating lymphocytes could explain some of the discrepancies between PB and BM results. In fact, Reinhold *et al.* (22) found a good correlation between BM karyotyping and PB FACS (fluorescence-activated cell sorter) sorted neutrophils. They showed also that the correlation between FISH and cytogenetics is weaker in patients on imatinib than in patients on non-imatinib therapies.

The sensitivity of interphase FISH is limited by the FP rate, i.e. the frequency with which BCR and ABL signals randomly co-localize in normal cells. In practice, the limit of detection of CML cells is typically 1–5% and depends in part on which probes are used, the size of the nucleus, the precise portion of the breakpoint within the ABL gene and the criteria used to define co-localization. The specificity of the newer split signal assay is higher with <1% FP (7, 21, 26). Recently, we have reported our experience with a novel system that provides combined morphological and FISH analysis on the same cells, using the BioView (Duet^{TM} BioView Ltd, Rehovot, Israel) system. This system allows rapid automatic scanning of a large number of cells for a cytogenetic abnormality. When a specific underlying malignancy is associated with a specific cytogenetic abnormality a specific FISH probe is used to identify the MRD population and the system relocates the morphology of the cells harboring this abnormality, thus increasing the specificity and sensitivity of the test (27). In the future it will be worth to use also this method for evaluation of MRD in these patients.

Currently, the most sensitive method to detect MRD after treatment for CML is RT-PCR. Like FISH, RT-PCR does not require dividing cells, so that PB can be utilized. Qualitative results obtained from PB have a good concordance with those from BM (7, 10, 11). The present multiplex assay used by us in this study is a rapid and practical qualitative method that uses three forward primers for all the major types of BCR–ABL rearrangement in a single reaction. Its sensitivity is 1 : 1000. Thus, multiplex RT-PCR can detect even one Ph-positive cell among 100 or 1000 normal cells in 1 μg of RNA. This level of sensitivity is clearly useful for the confirmation of clinical disease or the detection of preclinical relapse and is a little higher than that of FISH (28). For MRD detection, it has been claimed that RT-PCR is the most sensitive test. This has been challenged by a study of 21 patients who were in complete cytogenetic response. While FISH detected 1–12% BCR–ABL fusion genes in all of these patients, RT-PCR detected fusion mRNA transcripts in only six of them, suggesting the existence of transcriptionally silent BCR–ABL cells (29, 30). RT-PCR has a lower specificity compared with either cytogenetics or FISH because of cross-contamination of a negative specimen by a positive specimen. This limits its utility as a single diagnostic test for MRD follow-up (5).

Quantitative RT-PCR predicts better than qualitative RT-PCR imminent cytogenetic or hematological relapse. Thus the detection of an increasing level of leukemia burden may lead to early therapeutic interventions, even in patients who are still in

complete cytogenetic remission. The introduction of RQ-PCR made quantification of MRD more practical. It offers a dynamic detection range over five orders of magnitude and the real-time detection of amplicons minimizes the risk of contamination (5, 6, 8, 30, 31). The choice of a laboratory test for CML monitoring depends on the degree to which a particular therapy reduces the number of leukemic cells. Cytogenetics, FISH, and RT-PCR represent tests with increasing levels of sensitivity. A central principle in selecting a test for following a particular therapy is to match the sensitivity of the test to the therapeutic effectiveness. The same implies also for newer therapies like imatinib.

We compared the results obtained by multiplex RT-PCR and FISH in our patients and found an excellent correlation between the two methods with respect to the concordance of the positive and negative results, and the kinetics of the achievement of molecular remission. Similar results have been reported previously (27, 32).

Recently, several studies have evaluated the response to imatinib treatment in CML patients and compared between the cytogenetic and molecular response. These studies showed the same pattern: a very rapid decline in transcript numbers reaching a low but still measurable plateau. Unfortunately, so far only a small number of patients have achieved undetectable levels or BCR–ABL transcripts as assessed by RQ-PCR or two-step nested RT-PCR (9, 16, 18, 33–41). Recently, Hughes *et al.* (34) published the IRIS study results on the levels of BCR–ABL transcripts measured by RQ-PCR. They showed that in patients who achieved complete cytogenetic remission, levels pf BCR–ABL transcripts after 12 months of treatment had fallen by at least 3 log in 57% of patients treated by imatinib with an estimated 39% of all patients treated with imatinib having a reduction of at least 3 log in the BCR–ABL level. Patients who had a reduction in the level of BCR–ABL of at least 3 log had a negligible risk of disease progression over the subsequent 12 months (34).

We applied the RQ-PCR method on 78% of our negative multiplex samples and found that 28% of them were 'true' RQ-PCR negative, implying a rate of around 14% 'true negativity' (nine 'true negative' of a total of 65 multiplex tests). Notably, these nine results were obtained from seven patients, meaning that the rate of patients achieving 'true negativity' was even lower than 14%. Nevertheless, this rate is higher than that reported recently by Hughes *et al.* (34). They used very stringent criteria and showed that no BCR–ABL transcripts were detected on at least one occasion in 4% of patients treated with imatinib. This discrepancy can be attributed to the much smaller number of our cohort and to the fact that we did not use nested RT-PCR to establish 'true negativity'. This was not performed as the main aim of this study was to compare between the three methods and not to establish the rate of 'true negativity' in CML patients treated with imatinib. These results are in accordance with those reported in the literature so far for imatinib. Merx *et al.* (41) analyzed 486 peripheral blood samples in 120 patients with CML treated with imatinib on the German studies. They reported an excellent correlation between cytogenetic and RQ-PCR studies and found that the BCR–ABL/ABL ratio at 2 months predicted for the 6-month major cytogenetic response rate and that all complete cytogenetic responders were still PCR positive at the time of follow-up. Brandford *et al.* (42) found similar results in 38 patients with CML on imatinib therapy studied by them. Kantarjian *et al.* found a good correlation between cytogenetic and RQ-PCR studies and a good concordance between RQ-PCR values and cytogenetic response categories (9). Others have also reported good correlations between RQ-PCR values and cytogenetic or FISH studies and a rapid reduction of BCR–ABL transcript levels with imatinib therapy compared with IFN therapy (43–45). Our findings also support the conclusion that a good correlation exists between the various methods for evaluation of MRD during imatinib therapy in CML patients.

Recently, several groups reported on the emergence of clonal cytogenetic abnormalities in CML patients in cytogenetic remission to imatinib. The significance of these abnormalities is still controversial and it is still unknown whether they predict for disease progression or represented myelodysplastic changes secondary to the treatment with imatinib or whether they have no clinical consequences (46–50). Regardless of their origin, the occurrence of such changes implies that molecular follow-up with RT-PCR or RQ-PCR alone is not sufficient and intermittent monitoring by cytogenetics and or FISH analysis for the other abnormalities should be carried out.

It was hard for us to draw conclusions regarding the kinetics of molecular remission achievement in CML patients treated with imatinib because of the retrospective nature of our study. Yet, from our results and even from those reported in the literature recently a good molecular response should be achieved within 1 yr of treatment with imatinib.

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