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Early reduction of BCR-ABL mRNA transcript levels predicts cytogenetic response in chronic phase CML patients treated with imatinib after failure of interferon α

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The degree of tumor load reduction as measured by cytogenetic response is an important prognostic factor for chronic myelogenous leukemia (CML) patients on therapy. We sought to determine whether BCR-ABL transcript levels can predict chromosomal response. Residual disease was evaluated in 120 CML patients in chronic phase (CP) treated with the selective tyrosine kinase inhibitor imatinib after resistance or intolerance to interferon α (IFN). Median time of therapy was 401 days (range 111–704). BCR-ABL and total ABL transcripts were measured in 486 peripheral blood (PB) specimens with a real time RT-PCR approach using fluorescent-labeled hybridization probes (LightCycler technology) and results were expressed as the ratio BCR-ABL/ABL. Cytogenetic response was determined in 3-monthly intervals: From 101 evaluable patients, 42 achieved a complete (CR, 0% Philadelphia chromosome (Ph)-positive metaphases), 18 a partial (PR, 1–34% Ph+), 13 a minor (MR, 35–94% Ph+), and 26 no response (NR, >94% Ph+). All PB samples were RT-PCR positive. The proportion of Ph+ metaphases and simultaneous BCR-ABL/ABL ratios correlated with $r = 0.74$, $P < 0.0001$. In order to investigate whether early molecular analysis may predict cytogenetic response, quantitative RT-PCR data obtained after 1 and 2 months of therapy were compared with cytogenetic response at 6 months. BCR-ABL/ABL ratios after 1 month were not predictive, but results after 2 months correlated with the consecutive cytogenetic response ($P = 0.0008$). The probability for a major cytogenetic response was significantly higher in patients with a BCR-ABL/ABL ratio <20% after 2 months of imatinib therapy. We conclude that: (1) quantitative determination of residual disease with real time RT-PCR is a reliable and sensitive method to monitor CML patients on imatinib therapy; (2) BCR-ABL/ABL ratios correlate well with cytogenetic response; (3) in IFN-pretreated patients all complete responders to imatinib have evidence of residual disease with the limited follow-up available; and (4) cytogenetic response at 6 months of therapy in CP patients is predictable with real time RT-PCR at 2 months.
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Introduction

Chronic myeloid leukemia (CML) is characterized by the reciprocal translocation t(9;22)(q34;q11), which results in the formation of the Philadelphia (Ph) chromosome. The consequence of this translocation is a fusion of the BCR gene on chromosome 22 to the cABL gene on chromosome 9, resulting in a novel chimeric BCR-ABL gene, which is expressed as a fusion protein with deregulated tyrosine kinase activity that has been recognized to play the keyrole in the pathogenesis of CML.[1,2]

Imatinib (formerly STI571) is a selective inhibitor of the BCR-ABL protein tyrosine kinase. In a phase I dose-escalation study, daily doses of ≥300 mg imatinib induced durable hematologic responses in 53/54 CML patients in chronic phase (CP) pretreated with interferon α (IFN).[3] The efficacy of imatinib in these patients has been confirmed in a phase II study showing a major cytogenetic response rate of 60% after a median observation time of 18 months, 41% being complete.[4]

The degree of tumor load reduction, ie the residual amount of Ph+ cells, after therapy is an important prognostic factor for patients with CML after therapy with IFN.[1,5,6] The standard method to estimate the tumor burden in CML patients is conventional cytogenetic analysis of bone marrow (BM) metaphases.[5] However, chromosome banding analysis is labor-intensive, time-consuming, and expensive. The limited number of cells analyzed leads to a high variability of results.[7] The frequency of cytogenetic analysis can be reduced if CML patients are monitored by molecular methods like real time reverse transcriptase polymerase chain reaction (real time RT-PCR) for detection of BCR-ABL mRNA, which can be performed on peripheral blood (PB) specimens and are therefore less invasive than conventional metaphase analysis.[8–10] Furthermore, real time RT-PCR is a highly sensitive method, which can detect one CML cell in 10^5 normal cells.[9] Considering the high proportion of complete cytogenetic responders after imatinib therapy, 41% in IFN-pretreated CP patients,[4] the need for more sensitive methods to monitor residual disease becomes obvious.

In this study we have evaluated quantitative real-time RT-PCR as a method to monitor CML patients after therapy with imatinib. In order to elucidate whether early reduction of BCR-ABL mRNA levels could predict for consecutive cytogenetic response, RT-PCR results were compared with simultaneous and consecutive cytogenetic results.

Patients and methods

Patients

A total of 120 patients (69 male, 51 female) with CML in CP resistant or intolerant to IFN-based therapies and treated with imatinib in a single center were studied. Patients were recruited into two consecutive phase II protocols (multi-institutional studies 0110[4] and 0113, conducted by Novartis Pharma, Basel, Switzerland). Informed consent was obtained as required by the Declaration of Helsinki. Median age at start of imatinib therapy was 59.0 years (range 21.4–81.6). Median follow-up after start of imatinib therapy was 401 days (range 111–704). Patients received 400 mg imatinib p.o. with the option of dose adjustment according to the trial protocol.[4]

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Cytogenetic analysis

Cytogenetic analysis was performed on bone marrow (BM) aspirates according to standard protocols in 3-monthly intervals.[5] BM samples were processed within 24 h after aspiration. Cytogenetic response was evaluated as follows: complete response (CR), 0% Ph+ metaphases; partial response (PR), 1 to 34% Ph+ metaphases; minor response (MR), 35 to 94% Ph+ metaphases and non-response (NR), >94% Ph+ metaphases. Between three and 35 (median, 25) metaphases were analyzed. For comparison with quantitative RT-PCR data, only results obtained from >10 metaphases were considered. Between one and eight (median four) samples were investigated from each patient during follow-up periods up to 22 months.

RNA extraction, cDNA synthesis

Total leukocyte RNA was extracted from 10 to 40 ml PB after lysis of red blood cells. Samples were collected either locally or sent by mail and spent up to 2 days in transit. RNA extraction was performed using commercially available extraction kits as suggested by the manufacturer (RNeasy; Qiagen, Hilden, Germany) or, for sensitive detection of residual disease in complete cytogenetic responders, by CsCl gradient centrifugation. RNA was reverse transcribed using random hexamer priming and MMLV reverse transcriptase as published.[11]

Qualitative PCR

Prior to therapy *multiplex PCR* was performed to determine the type of the BCR-ABL fusion transcript as described previously.[12] In two patients with CR to IFN prior to imatinib therapy, *nested RT-PCR* was performed in order to allow sensitive detection of BCR-ABL.[13]

Quantitative real time RT-PCR

PB samples were collected monthly during the first 6 months of imatinib therapy and afterwards in 3-monthly intervalls. Up to 13 samples were investigated from each patient. BCR-ABL transcripts were quantified by real time RT-PCR using the LightCycler technology as described.[9,14,15] Conditions were established to amplify <10 target molecules per reaction and to detect one CML cell in 10^5 cells from healthy donors.[9,10] A standard curve for each run was constructed and the number of target molecules in each sample was calculated by reference to this curve. To control variables in the quality of RNA total ABL transcripts were quantified and results were expressed as BCR-ABL/ABL ratio (in percentage). If real time PCR revealed <10 BCR-ABL transcripts/reaction nested RT-PCR was performed[13] and the final result expressed as the ratio <10 BCR-ABL divided by the number of control gene transcripts in the individual sample. Strict precautions were taken to prevent contamination. All experiments included negative controls from all stages of the reactions.

Statistical analysis

The correlation between the percentage of Ph+ metaphases and contemporaneous BCR-ABL/ABL ratios was evaluated using Spearman's rank correlation coefficient. Comparison of BCR-ABL/ABL ratios and cytogenetic response groups was made using the Kruskal–Wallis and Mann–Whitney tests.

Results

Qualitative determination of the BCR-ABL fusion

Prior to imatinib therapy, 74 patients (61.7%) expressed b3a2, 37 patients b2a2 (30.8%), eight patients both b3a2 and b2a2 (6.7%), and one patient e19a2 mRNA transcripts (0.8%).

Cytogenetic response

From 118 Ph-positive patients at diagnosis, cytogenetic response to the pre-treatment with IFN was prior to the start of imatinib none ($n = 102$), minor ($n = 14$), and complete ($n = 2$). Two patients were Ph-negative/BCR-ABL-positive at diagnosis. From 101 evaluable patients with at least one cytogenetic analysis in follow-up, best cytogenetic response to imatinib (including prior response to IFN) was CR ($n = 42$, 41.6%), PR ($n = 18$, 17.8%), MR ($n = 13$, 12.9%), or none ($n = 26$, 25.7%) with a median duration of therapy of 401 (111–704) days.

Comparison of quantitative PCR and cytogenetics

A total of 486 PB samples from 120 patients were analyzed. The correlation between the BCR-ABL/ABL ratio and the percentage of Ph+ metaphases in those samples who had contemporaneous analysis of both methods ($n = 295$) was $r = 0.73$ ($P < 0.0001$).

Real time RT-PCR revealed residual disease in 474/486 samples (97.5%). In 12 samples (2.5%) quantification determ-

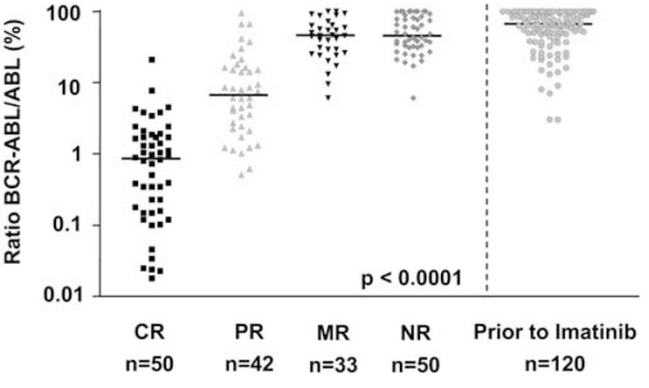

Figure 1 Comparison of the BCR-ABL/ABL ratio derived from real-time RT-PCR of peripheral blood specimens *vs* cytogenetic analyses of bone marrow metaphases. BCR-ABL/ABL ratios of patients achieving a complete, partial and minor cytogenetic remission differed significantly. There was no difference between minor and non-responders.

Table 1 Molecular response according to duration of imatinib therapy

Duration of imatinib therapy (months)	*0*	*1*	*2*	*3*	*4–6*	*7–9*	*10–12*
n	77	55	30	74	93	40	18
BCR-ABL/ABL ratio							
median (%)	74	45	22	36	17	20	0.56
range (%)	3.8–100	3.1–100	1.4–100	0.18–100	0.016–100	0.034–100	0.0080–67

Only patients with 100% Ph-positive metaphases prior to therapy were considered.

ined <10 transcripts/reaction and nested RT-PCR was additionally performed which was positive in all cases.

BCR-ABL/ABL ratios differed significantly between the four cytogenetically defined response groups ($P < 0.0001$, Kruskal–Wallis test). Median BCR-ABL/ABL ratio at start of imatinib therapy was 67.0% (range 0.01–100%, $n = 120$). Median ratio of samples from patients in CR was 0.85% (0.018–21%, $n = 50$), in PR 6.7% (0.5–94%, $n = 42$), in MR 45% (6.0–100%, $n = 33$), and in NR 45.5% (6.0–100%, $n = 50$). There was a significant difference between the median BCR-ABL/ABL ratios of patients in CR and PR ($P < 0.0001$) and between PR and MR ($P < 0.0001$), but not between samples of patients in MR or NR (Figure 1).

Whilst individual molecular response within the first year of treatment spanned a range over four orders of magnitude, median BCR-ABL/ABL ratios decreased gradually (Table 1).

Prediction of cytogenetic response

In order to investigate whether early molecular analysis may predict cytogenetic response, quantitative real time RT-PCR data achieved after 1, 2 and 3 months of therapy were compared with cytogenetic response at 6 months. From 60 evaluable patients lacking any cytogenetic response prior to imatinib treatment, 16 achieved a CR (26.7%), 18 a PR (30.0%), 12 a MR (20.0%), and 14 patients NR to imatinib after 6 months of therapy.

BCR-ABL/ABL ratios after 1 month of therapy were not predictive for consecutive cytogenetic response at month 6 (median 29.0% for CR, 49.0% for PR, 50.0% for MR, and 52.0% for NR; NS) However, median BCR-ABL/ABL ratios at month 2 predicted CR at month 6 (median 4.7% for CR, 31.0% for PR, 40.0% for MR, and 74.0% for NR; $P = 0.0008$, Table 2 and Figure 2).

The probability for a major cytogenetic response (CR plus PR) after 6 months of imatinib therapy was significantly higher in patients achieving a BCR-ABL/ABL ratio <20% at 2 months ($P = 0.007$, Figure 3).

Table 2 Comparison of median ratios BCR-ABL/ABL at 1, 2 and 3 months after treatment with imatinib and further cytogenetic response after 6 months of imatinib therapy

Cytogenetic response	*CR*	*PR*	*MR*	*NR*	
At month 6 *n*	16	18	12	14	
BCR-ABL/ABL Ratio (%):					
Month 1	29	49	50	52	NS
Month 2	**4.7**	31	40	74	$P = 0.0008$
Month 3	**2.5**	8.0	40	38	$P < 0.0001$

CR, complete; PR, partial; MR, minor; NR, non-response.

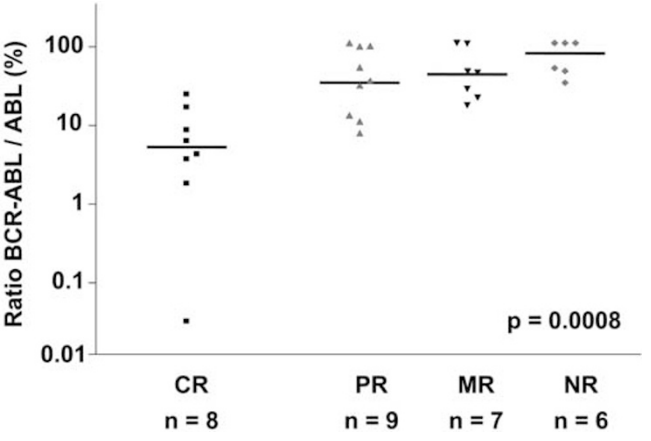

Figure 2 BCR-ABL/ABL ratios quantificated by real-time RT-PCR of peripherial blood 2 months after start of imatinib therapy according to cytogenetic response after 6 months of therapy. BCR-ABL/ABL ratios at 2 months of patients achieving a complete cytogenetic response after 6 months of therapy differed significantly from patients achieving a partial, minor or non-response.

Discussion

The use of the selective tyrosine kinase inhibitor imatinib for the treatment of CML in CP resulted in rapid hematologic and cytogenetic responses in the majority of patients.[3,4] Cytogenetic assays continue to be regarded as the standard method for assessing response to therapy in CML. However, molecular methods of response determination, such as RT-PCR, are highly sensitive, quantitative, and can be performed on PB specimens that may allow easier and more frequent monitoring.[8,16,17] Quantitative RT-PCR methods have been evaluated to estimate residual disease and predict relapse after allogeneic[13,18,19] or autologous stem cell transplantation,[20,21] and IFN therapy.[6,22] The level of BCR-ABL fusion mRNA at single-time points correlates well with stage of the disease[13] and cytogenetic response.[9,22,23] On serial analyses, patients with increasing levels of BCR-ABL have a greater probability of relapse.[24] However, the applicability of these methods to patients on imatinib therapy and the predictive value of their results has not been studied so far in a large cohort of patients.

Our analysis of 486 PB samples from 120 patients with CML in CP treated with imatinib after IFN failure clearly shows a good correlation of cytogenetic response data with quantita-

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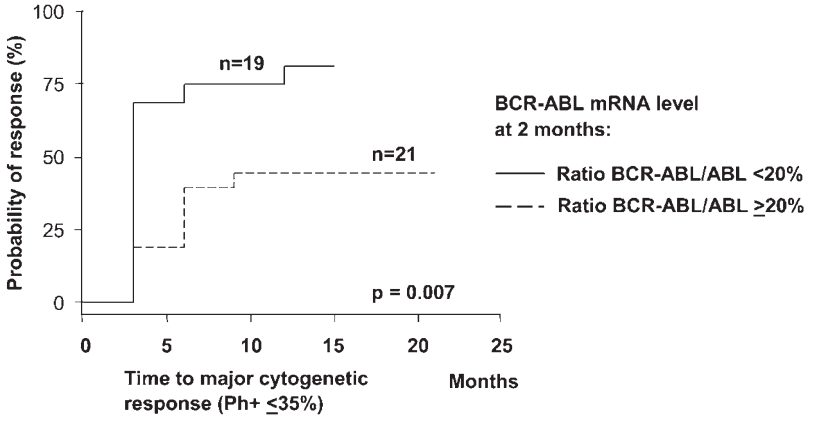

Figure 3 Probability of a major cytogenetic response (Ph+ ≤35%) by BCR-ABL/ABL ratios 2 months after start of imatinib therapy. For patients with a BCR-ABL/ABL ratio <20% at month 2 the probability of a major cytogenetic response was significantly higher than for those patients with a BCR-ABL/ABL ratio ≥20%.

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tive RT-PCR results. This finding could contribute to a more frequent analysis of residual disease on long-term treatment and reduce the frequency of BM aspirations.

All complete cytogenetic responders to imatinib continued to express BCR-ABL transcript in PB. In contrast, RT-PCR negativity was reported in one out of 54 patients of the imatinib phase I trial.[3] This discrepancy may be due to different technologies used. In addition to RT-PCR strategies, which are widely comparable[25] and standardized,[26] preanalytical conditions such as the amount of PB and number of leukocytes processed, methods of RNA extraction and cDNA synthesis are considered crucial for the final sensitivity of the assay. Thus, we analyzed cell lysates derived from 20 ml PB corresponding to 6×10^7 nucleated cells in a patient in good hematologic response with leukocyte counts of 3 000/μl.

The actual level of residual disease in CR spans a range over three orders of magnitude and parallels data obtained after IFN therapy.[6] However, considering the short observation time and the low frequency of hematologic relapse in patients treated with imatinib,[4] the prognostic significance of the level of residual disease cannot be determined as yet. Only one patient with progressive disease due to clonal selection of cells with a mutation of the ATP binding site was observed out of 42 patients in CR.

Conflicting results comparing MRD data on the cellular (FISH) and genomic (Southern blot analysis) level were reported,[27,28] suggesting the existence of transcriptionally silent BCR-ABL cells in a significant proportion of responders to IFN and allogeneic stem cell transplantation. However, these data could not be verified by others.[5,29–31] In 29 patients in CR to imatinib, we did not observe a significant proportion of FISH-positive cells in interphase preparations.[32]

In agreement with data achieved in patients after IFN therapy,[9,22] BCR-ABL/ABL ratios and contemporaneous cytogenetic analyses correlated well. Prior to the first cytogenetic evaluation after 3 months of therapy, a significant proportion of patients showed a rapid reduction of BCR-ABL transcripts. The occurrence of a complete or major CR after 6 months could be predicted from the dynamics of BCR-ABL transcripts within the first 2 months of therapy. Assuming that reduction of the tumour burden on imatinib has the same prognostic value as cytogenetic and molecular response to IFN[33,34] or stem cell transplantation,[13,24] this finding could contribute to an early individual tailoring of therapy (dose increase, combination therapies, alternative treatments, eg allogeneic stem cell transplantation).

We conclude, that real time RT-PCR is a sensitive and reliable tool to monitor patients after therapy with the selective tyrosine kinase inhibitor imatinib. All patients on second line therapy after failure to IFN have evidence of minimal residual disease. Molecular response at 2 months predicts consecutive cytogenetic response and therefore may help to optimize therapy and monitoring. The expected proportion of patients in CR requires the urgent need for international adjustment of preanalytic conditions and RT-PCR strategies in order to standardize results obtained in different laboratories and to allow a worldwide comparable assessment of molecular response to imatinib.

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