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ARTICLE

Correlation Between BCR-ABL Expression and Tumor Burden is Restricted to the Transition from Minor to Major Cytogenetic Response in Interferon Treated CML Patients

"A pre–Gleevec study"

László KERESKAI, János A VASS, Mária KNEIF, László PAJOR

University of Pécs, Faculty of Medicine, Department of Pathology

The interferon treatment of chronic myeloid leukaemia has been monitored by investigating the tumour burden as revealed by fluorescence in situ hybridization and the expression of BCR-ABL chimera determined by quantitative reverse transcription polymerase chain reaction. These parameters were obtained from the peripheral blood of 51 untreated and 104 follow-up patient samples. Poor corrrelation (r = .31) was found between BCR-ABL expression and tumor load in all samples as well as in untreated patients, and this correlation was even less in all follow-up cases (r = .28). Regarding chimera expression five order of magnitude difference existed in the untreated patients and this value dropped to two in those with complete cytogenetic response. Only the major and the complete cytogenetic response groups differed significantly (p .001) in the BCR–ABL expression from that of patients at diagnosis. Among the different cytogenetic response groups the only significant difference (p<.01) in the BCR-ABL expression was obtained between the major and the minor responders. In the individual patients not only correlated changes of residual tumour mass and chimera expression, but mainly independent changes of these two parameters were observed. This indicates that the BCR-ABL expression and the tumor burden are largely independent variables. (Pathology Oncology Research Vol 9, No 3, 174–179)

Keywords: chronic myeloid leukaemia, molecular monitoring, quantitative polymerase chain reaction, fluorescence in situ hybridisation

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Correspondence: László PAJOR, University of Pécs, Faculty of Medicine, Department of Pathology, 12 Szigeti Str, H-7624 Pécs, Hungary; Phone: +36 72 536-282, Fax: +36 72 536-281, e-mail: pajor@pathology.pote.hu

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List of abbreviations

Bcr: breakpoint cluster region, bcr-abl: chimera of bcr and abl gene, BMT: bone marrow transplantation: CCA: conventional cytogenetic analysis; cDNA: copy DNA; CML: chronic myeloid leukemia; FISH: fluorescence in situ hybridization; IFN: interferon; IPC: interphase cytogenetics; M-bcr: major bcr; MMLV-RT: Maloney Murine Leukemia Virus Reverse Transcriptase; PCR: polymerase chain reaction; Ph: Philadelphia chromosome; Q-PCR: quantitative reverse transcription polymerase chain reaction; RT: reverse transcription

Introduction

The chronic myeloid leukemia (CML) phenotype is evoked and mantained by the expression of the BCR-ABL chimera gene which, however, represents a broad range.[13,21,26,29] One extreme of this the silent Philadelphia (Ph) chromosome. This non-expressing fusion gene might exist not only in individual haemopoietic colonies, but - under special circumstances – at the entire clonal haemopoiesis level.[1,3,20,25,31,33] The tumor load, on the other hand, is represented by the ratio of cells harbouring the BCR-ABL rearrangement among the haemopoietic elements. Ultimate cure of CML which originates from uncommitted stem cells can only be expected from allogeneic bone marrow transplantation (BMT) which is, however, only available for a fraction of the patients.[17] One of the choices for the majoring of the patients is the α-interferon (α-IFN) treatment which has an anti-prolifer-

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ative, differentiation inducing capacity, and its inhibitory effect on BCR-ABL expression is well documented.[7,27,28]

For the molecular monitoring of the efficiency of any, thus the α-IFN treatment, one needs to quantify both the tumour load and the chimera gene expression. For the first we determined the percentage of the BCR-ABL rearrangement positive cells in the peripheral blood by fluorescence in situ hybridization (FISH) and interphase cytogenetics (IPC), whereas the other parameter was obtained by quantitative reverse transcription polymerase chain reaction (Q-PCR). Our study we have intended to reveal the correlation between the two parameters in untreated patients and especially upon treatment, since currently only the tumor load as defined by the percentage of the Ph positive cells is considered in the estimation of the therapeutic response.[19,30] Furthermore, based on the available data the correlation between tumor load and chimera expression is not thoroughly defined.[6,11,14,21,29]

Materials and Methods

Patients

The investigations included 155 peripheral blood samples of 68 CML patients, sent for molecular analysis on behalf of the Hungarian Myeloproliferative Study Group to a reference molecular pathology laboratory. Out of the 155 samples 51 derived from patients at diagnosis (untreated patients), who were in chronic phase according to the published criteria,[30] whereas 104 originated from α-IFN treated cases. α-IFN was administered according to international guidelines and the treatment was preceeded by cytoreduction achieved by hydroxyurea.[18] The average duration of the follow-up periods was 14 months (range: 4 to 35 months).

RNA isolation

Total cellular RNA was isolated from $2x10^7$ cells collected from the buffy coat of blood samples anticoagulated by EDTA. The isolation was accomplished by means of Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instruction.

Reverse transcription (RT)

Two different RT reactions were performed. The RT needed for the qualitative M-bcr PCR reaction (a2-RT)) was made in a 10 µl volume in the presence of 2.4 U MMLV-RT enzyme (Maloney Murine Leukaemia Virus Reverse Transcriptase; Appligene-Oncor, France), 2 µl of the 5X reaction buffer (Appligene-Oncor, France), 500 µmol/l of each dNTPs (Life Technologies, Gaithersburg, MD), 60 µg RNA and 15 nmol/l of the first step antisense primer (a2) of the M-bcr PCR reaction , at 37 °C for 60 minutes. The RT (Q-RT) for the quantitative PCR (Q-PCR) was performed in 30 µl volume in the presence of 7.2 U MMLV-RT enzyme, 6 µl of 5X reaction buffer, 500 µmol/l of each dNTPs, 180 µg RNA as well as 15 nmol/l of the antisense primer (a3) of the abl 2–3 PCR, at 37 °C for 60 minutes.

M-bcr qualitative polymerase chain reaction

The molecular diagnostics, the determination of the b3/a2, b2/a2 or mixed type of chimera RNA were accomplished by an M-bcr nested PCR reaction according to Gaiger et al.[8] The quality of the isolated RNA was checked by amplification of the abl 2-3 exons as described by Hermans et al.[10]

Quantitative polymerase chain reaction (Q-PCR)

The BCR-ABL expression was defined as the ratio of the amount of BCR-ABL as well as the abl 2-3 transcripts which were determined in separate Q-PCRs. The BCR-ABL and the abl 2-3 mRNAs were transcribed into cDNA in one reaction (Q-RT) in order to minimize the inaccurancy due to the variation in the efficiency of the different RT reactions.

Competitors

The b2a2 or the b3a2 type of the first step amplification product from the M-bcr reaction was used as competitor for the quantitation of the BCR-ABL expression in patients samples harbouring either the b3a2 or the b2a2 type of translocation. The first competitor was obtained from a patient sample, the other one from the K-562 cell line. The following synthetic competitor was used for the quantitative abl 2-3 PCR: 5'-CAG CGG CCA GTA GCA TCT GAC TTT GAG CCT CAG GGT CTG AGT GAA GCC GCT CGT TGG AAT TCC AAG GAA AAC CTT CTC GCT GGA CCC GTG AAA AGC TCC GGG TCT TAG GCT ATA ATC ACA-3'. This oligonucleotide and all other primers were obtained from Integrated DNA Technologies, Inc., Coralville, IA. The patient samples were amplified using the M-bcr as well as the abl 2-3 PCR in the presence of the appropriate competitor at the concentration of 0.01–1000 fM using a logaritmic (=√10) dilution series. The equivalence point between different competitor concentrations was determined.

Interphase cytogenetics (IPC)

The BCR-ABL rearrangement was investigated on interphase nuclei using P1 clones (Vysis Inc, Downers Grove, IL). White blood cells from the peripheral blood were fixed in 70% ethanol, postfixed in 50% acetic acid and airdried on slides. FISH was performed on these preparations according to the manufacturer's instructions.

Table 1. **The range of BCR-ABL transcript number as well as the ratio of the BCR-ABL vs abl transcript numbers in the different cytogenetic response groups and in patients at diagnosis.**

Cytogenetic response	*No of patients*	*Transcript/μg RNA*	*Ratio (%)*
Complete (0%)	12	20-1590 (431; 508)*	0.025-1.26 (0.28; 0.34)*
Major (1-33%)	29	20-7960 (1150; 1560)	0.050-6.31 (1.18; 1.59)
Minor (34-66%)	36	16-126000 (11500; 25000)	0.010-31.6 (5.28; 8.24)
Nonresponder (>66%)	27	16-252000 (20000; 51800)	0.016-63.1 (6.75; 13.5)
At diagnosis	51	40-1000000 (96500; 201000)	0.079-100 (20.0; 37.8)

*Mean and standard deviations are given in parenthesis.

After hybridization and development the preparations were mounted with DAPI/Vectashield (Vector Laboratories Inc., Burlingame, CA) and investigated in a Zeiss Axioskop epi-illumination fluorescence microscope equipped with VY-DGO triple bandpass filter (Vysis Inc). At least 200 cells with intact nuclear morphology were evaluated per patient sample. The cells exhibiting the yellow fusion signal were taken as positive for the rearrangement. The false positivity +2 SD value proved to be 4.8% on white blood cells of healthy donors, therefore a 5% cut-off level was determined in this study. The following cytogenetic response categories were set: non-responders (66% < Ph+ cells), minor response (33% < Ph+ cells ≤ 66%), major response (1% ≤ Ph+ cells ≤33%), complete response (no Ph+ cells).[30] Considering the cut-off level, 1% true positivity implies 6/100 nuclei with fusion signal and the complete response less than 6 nuclei with fusion signal out of 100 nuclei.

Statistical Analysis

Mean, standard deviation, linear regression, correlation and t-probe were obtained by the Microsoft Excel software version 5.0a.

Results

Qualitative PCR

A total of 155 peripheral blood samples from 68 BCR-ABL rearrangement positive CML patients were analyzed by RT-PCR for BCR-ABL expression. All the samples proved to be BCR-ABL positive except one follow-up sample, the patient was, however, positive at the onset of the disease. Only the patients expressing either the b3/a2 or the b2/a2 but not both were included into this study. One patient expressing b3/a2 at the onset of the disease turned into mixed type of expression during the follow-up period and was excluded from the study. Out of the 68 patients 33 (48.5%) expressed the b2/a2 whereas 35 (51.5%) the b3/a2 type of transcript. No significant difference was found in the distribution of the two expression types among the cytogenetic response groups, except those achieving complete cytogenetic response where the b2/a2 type of expression was five times more frequent than the b3/a2 one (10 vs 2 patients).

Cytogenetic analysis

FISH analysis and results were available from all the 155 samples. The 51 patients at diagnosis had on average 76% + / – 11% Ph positive cells. The distribution of the follow-up samples among the cytogenetic response groups was as follows: complete (n = 12), major (n = 29), minor (n = 36), non-responders (n = 27).

Quantitative PCR vs cytogenetics

The lowest detectable BCR-ABL concentration in this assay was 0.01 fM, the bcr-abl transcript number / μg RNA in all samples ranged from 16 to 1 000 000 (mean: 38 200). The lowest concentration of the ABL transcripts still detectable by PCR proved to be 3 fM, the expression in all samples ranged from 1260 to 3 990 000 (mean: 317 000) transcript number / μg RNA. All but one sample proved to be BCR-ABL+. In the single negative follow-up sample the ABL could be well amplified, thus this sample was considered as true PCR negative under our conditions (0.01 fM sensitivity threshold for BCR-ABL). The ABL transcript number was determined as internal standard and the BCR-ABL expression was also characterterized as the ratio of the BCR-ABL as well as the ABL transcript numbers in 1 μg total RNA. Instead of separate RT reactions the BCR-ABL and the ABL sequences were transcribed into cDNA in one RT reaction which reduced the variation of the BCR-ABL / ABL ratio calculation from 150% to 20%. The BCR-ABL / ABL ratio ranged in all samples from 0.01% to 100%, but the sensitivity of the system could have allowed the detection of a ratio as low as 0.0004%.

The BCR-ABL expression in term of transcript number exhibited a broad range in the individual cytogenetic response groups. There was, however, a decreasing tendency with 5, 4 and 2 order of magnitude differences

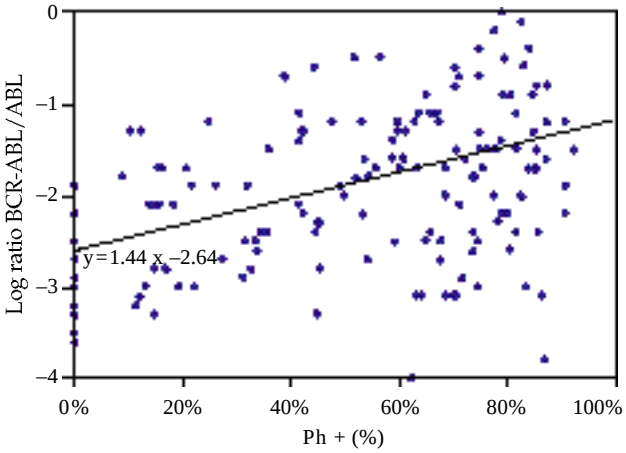

Figure 1. Correlation between interphase cytogenetic results and expression of BCR-ABL shown as the ratio of the BCR-ABL / ABL transcript numbers in all 155 patient samples.

between the minimum and maximum values in the untreated patients, then in the non– and minor responders as well as in the major and complete response groups, respectively *(Table 1).* When the percentage of the Ph+ interphase nuclei was compared with the BCR-ABL transcript number or the ratio of the BCR-ABL / ABL in all samples poor correlations were found (r = .30 and r = .31, respectively) (*Figure 1*). The same levels of correlations existed in the patient samples at diagnosis between the percentage of the Ph+ nuclei and the BCR-ABL expression (r = . 35 and r = .34, respectively). This low level of correlation even decreased in all follow-up samples (r = . 24 and r = . 28). Investigating the changes of the two parameters at the individual patient level, the optimal, i.e., the considerable decrease of both values was observed only in a fraction of the patients. In the majority of the patients all other variables occurred, i.e., with unchanging tumor burden (no cytogenetic response) a significant decrease in the expression of BCR-ABL (e.g., drop of ratio from 100% to 0.08%) was seen, whereas in other patients the values changed the other way around (*Figure 2*). Whatever was the cytogenetic response, no increase in the expression was observed in any patient upon treatment.

When the BCR-ABL expression in the untreated patient samples was compared with that in the different cytogenetic response groups the non-responder and minor-responder groups did not show considerable changes, wheras ($p > 0.01$), only the major and the complete cytogenetic response groups differed significantly ($p < 0.001$) in this value from that of the patients at diagnosis (*Figure 3*)**.** Furthermore, in the BCR-ABL expression the only significant difference among the cytogentic response groups was found between the major and the minor responders ($p < 0.01$), but not between the non-responders and the minor response groups as well as between the major and complete response groups, respectively.

Discussion

In this study we have analysed 51 untreated and 104 IFN treated follow-up patient samples for BCR-ABL expression and tumor burden in order to reveal the relationship between these two parameters. For the first one a competitive quantitative RT-PCR was used and the BCR-ABL/ABL ratio was determined. This quantitative PCR assay is different from what has first been introduced for this purpose, but the basic principles are the same[17]. The tumor load was defined as the percentage of BCR-ABL rearrangement positive cells among the peripheral white blood cell nuclei. We have chosen the FISH-IPC instead of the conventional cytogenetic analysis (CCA) because the sampling error is smaller by the first technique, it is not influenced by the clonal selection as occurs during culturing the cells and detects also molecular rearrangement.[2,16,24] Good correlations between results obtained by FISH-IPC and CCA in CML were reported implying that the relative tumor burden is well characterized by both techniques.[4,29] However, one analyses only partially overlapping cell populations by these two approaches, therefore the actual figures obtained by the two techniques might be different.[5,9,11,32] We have chosen blood samples for this analysis because this is the most easy and common way to monitor the disease and good correlation between cytogenetic data of blood and bone marrow samples have been reported in CML patients.[23]

The corrrelation of these two parameters is an important issue in monitoring treatment of CML patients. On the one hand, the BCR-ABL chimera product is known

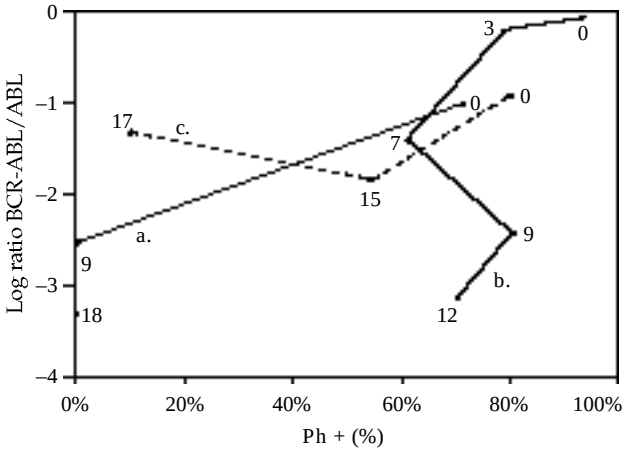

Figure 2. Changes of tumor burden and expression of BCR-ABL in three α-IFN treated patients. Figures at the individual dots indicate follow-up period in months, 0 indicates patient at diagnosis. Note that beside the response where both variables exhibited a considerable decrease (a), the tumor load (b) or the BCR-ABL expression (c) remained apparently unchanged, with a dramatic change of the other parameter.

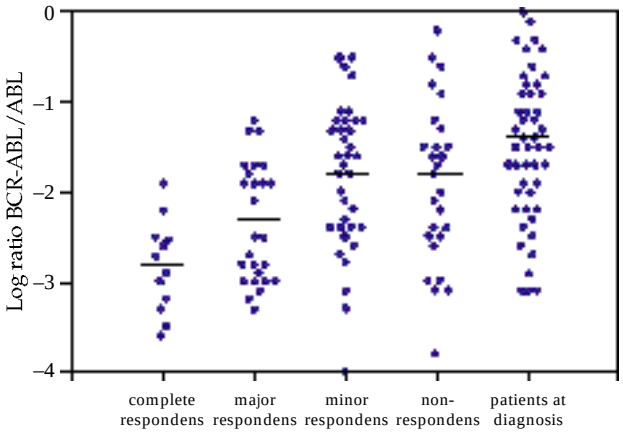

Figure 3. The distributions of the BCR-ABL / ABL ratio values and the means in the different cytogentic response groups as well as in patients at diagnosis. Only major and complete responders differed significantly ($p < 0.001$) from patients at diagnosis as well as from the rest of α-IFN treated ones.

to interact with the stem cell factor receptor p145 resulting in abrogation of the negative growth stimuli in a dose dependent way.[15,22] Thus, the measuring of the BCR-ABL expression represents quantification of the actual malignant potential of the disease. On the other hand, the determination of the tumor burden at the onset of the disease and upon treatment is a key element of any cancer managment protocol. Furthermore, quantifying the BCR-ABL expression by PCR will expand the approximately 1% to 5% sensitivity of the conventional FISH-IPC to a six order of magnitude range. Although residual disease at the level of 10^{-6} might not be the basis of major clinical decision making, a difference of five order of magnitude in the concentration of an aberrant molecule rendering the cells to proliferate can certainly not be disregarded.

We are aware of five publications deal which with the monitoring of α-IFN treated CML patients using both quantitative PCR and cytogenetics simultaneously. Four of the publications used CCA, one both CCA and FISH-IPC were applied. In two of these publications the expression was quantified only in CML patients with complete cytogenetic response.[12,13] The lower extreme of the expression in our patients with complete cytogenetic response was well comparable with the published ones, but we experienced only two instead of their four order of magnitude difference in expression in this cytogentic response group. In another publication BCR-ABL expression level was not statistically compared in the different cytogenetic response groups, but analysis of individual patients revealed no correlation between cytogenetic response and Q-PCR data in the majority of the cases.[21] In the remaining two studies cytogenetic response groups were set on the basis of bone marrow metaphase and interphase data according to Kantarjian et al which is different from what was used in our study.[11,19,29] In a FISH-IPC study with a 76% +/– 11% Ph+ nuceli at the onset of the disease the 94% threshold between non-responders and minor responders is hardly applicable. As the percental Ph positivity of our patients at diagnosis was presented in the upper one third of the range, we defined minor and major response when these values ranged in the middle and lower one third, respectively. For these reasons, it is hard to compare our data with the last two referred ones which claim that significant differences in BCR-ABL expression existed between the cytogenetic response groups except between untreated patients and non-responders in one study and between partial (less then 35% Ph+ cells) and complete responders in the other one. In our collection the non and the minor responders did not, while the major and complete responders differed significantly from patients at diagnosis in the BCR-ABL expression. Furthermore, the major and complete responders exhibited significant difference in the BCR-ABL/ABL ratio from minor and non-responders, but none of the first and the last two from each other. This implies that obtaining that cytogenetic data from the peripheral blood and by FISH-IPC the α-IFN treated CML patients can be grouped in term of BCR-ABL expression only into two categories: with and without at least major cytogenetic response. These data might be in concordance with the previously published ones that only major cytogenetic response was associated with significantly longer survival of α-IFN treated CML patients.[19]

We have found poor correlation between the number of Ph+ cells and BCR–ABL expression in both untreated and treated patients, which is in contrast to two publications and confirms another one.[11,21,29] Lack of correlation is, however, not suprising because the many orders of magnitude difference in BCR-ABL expression, the positive correlation between BCR-ABL expression and differentiation of myeloid cells, furthermore, the non-expressing, silent state of the Ph+ cells are well documented.[1,3,20,25,31,33] Independent changes of the two parameters are also clearly indicated at individual patient level where, in addition to correlated changes in some cases, all other variations of the two parameters were seen in the majority of the α-IFN treated patients.

In summary our conlcusions are as follow: *i.* BCR-ABL expression and tumor burden do not correlate in untreated patients and changes occur independently upon α-IFN treatment, *ii.*others than only major cytogenetic response are not predictive in term of a significant decrease of the BCR-ABL expression, *iii.* this is the largest collection of untreated and α-IFN treated CML patients investigated for BCR-ABL expression by quantitative PCR and tumor burden by interphase cytogenetics.

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