ORIGINAL ARTICLE

Specific assessment of *BCR–ABL* transcript overexpression and imatinib resistance in chronic myeloid leukemia patients

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Abstract

Imatinib mesylate has proven to be the most effective treatment in chronic myeloid leukemia. Nevertheless, imatinib resistance has raised concern and prompted interest in additional strategies to achieve disease eradication. Resistance to imatinib is mainly associated with three mechanisms: acquired mutations in the kinase domain of *BCR–ABL* protein, genetic amplification, and transcript overexpression of *BCR–ABL* rearrangement. Therefore an accurate assessment of resistance mechanism is particularly important to improve strategies to overcome resistance. In order to determine overexpression of *BCR–ABL*, we propose a method that correlates quantitative real time PCR and fluorescence *in situ* hybridization data from the same peripheral blood sample. The ratio between both methodologies permits to calculate the expression index (EI) for each patient. EI estimates the rate of *BCR–ABL* transcription *per* rearrangement. The median EI value, including all cases ($n = 123$), was 0.288; those cases ($n = 13$) included in percentile 90 showed an increment of EI above 1 Log (>2.88) with respect to the median value and were considered as cases with overexpression. We also evaluated the EIs using receiver operating characteristics curve; choosing an EI cutoff of 1.836 we obtained a sensitivity of 95% and a specificity of 61%. Using this EI cutoff value, more patients ($n = 17$) were included in the overexpression group. Patients within this group were resistant to imatinib and also showed a worse overall survival if compared with the remaining.

Chronic myeloid leukemia (CML) is characterized by the presence of the Philadelphia translocation $t(9; 22)(q34; q11)$, resulting from the fusion of the *BCR* and *ABL* genes (1, 2). The *BCR–ABL* fusion gene encodes a constitutively active tyrosine kinase leading to an enhanced proliferation of the affected cell clone. In recent clinical trials, the introduction of imatinib mesylate (formerly STI-571, Novartis Pharma, Basel, Switzerland), a compound that inhibits the tyrosine kinase activity of *BCR–ABL* (3), has shown excellent results in patients with CML, effective in reducing the burden of leukemia (4). Nevertheless, imatinib resistance and molecular evidence of persistent disease have raised concern and prompted interest in additional strategies, to

achieve disease eradication without the need for a stem cell transplant. The presence of several point mutations in the *ABL* kinase domain in relapsing patients after i-matinib therapy, resulting in reduced drug binding, is a major mechanism of acquired resistance to imatinib in CML (5, 6). Other mechanisms implicated as a cause of imatinib resistance include amplification of the *BCR–ABL* gene (7), overexpression of *BCR–ABL* transcripts (8) and activation of non-*BCR–ABL*-dependent transformation mechanisms (9, 10). Disease relapse and progression are associated with increased levels of *BCR–ABL* expression and acquisition of additional genetic and epigenetic abnormalities, which lead to altered hematopoietic cell growth and differentiation (11).

For the diagnosis and followup of CML patients, using fluorescence *in situ* hybridization (FISH) with probes for *BCR* and *ABL*, the standard Philadelphia translocation can be directly visualized in interphase nuclei as well as on metaphases (12). Interphase-FISH (IP-FISH) is useful to quantify the percentage of Philadelphia-positive nuclei and eventually characterize *BCR–ABL* gene amplification as resistance mechanism. Due to the higher number of analyzable nuclei, the sensitivity (0.5–1%) is increased compared with classical cytogenetics (5–10%) and also quantification becomes more exact. Moreover, recognition does not depend on the presence of the typical Ph chromosome, so that it is possible to detect cryptic translocations and the rare *BCR* and *ABL* fusion variants. In this context, the introduction of techniques for identifying and measuring *BCR–ABL* transcripts, has enabled more precise assessment of response to specific therapies for CML. RT-PCR based techniques are about 1000–10 000-fold more sensitive than FISH in picking up residual CML cells (13). Quantitative real-time PCR procedure allows a simple, reliable, and sensitive quantification of *BCR–ABL* transcripts (14). Therefore, serial measurement of *BCR–ABL* specific transcript is a valuable approach to monitor individual patients and in some cases to indicate the need to reassess therapy. As shown by the IRIS group study, it is assumed that in patients receiving imatinib a durable response can be achieved even without disease eradication if there is a reduction in levels of *BCR-ABL* transcripts of at least 3 Logs (4).

Despite a general good correlation between FISH and quantitative real time PCR (qRT-PCR) data (15), for a fraction of CML patients it has been reported that no significant relationship exists between the percentage of positive nuclei (by FISH) and *BCR–ABL/ABL* ratios (by qRT-PCR) (16). While for FISH-positive and qRT-PCR-negative cases several hypotheses have been formulated, such as that the *BCR–ABL* rearrangement persists unexpressed, the real significance of FISH-negative (or very low percentage of positive nuclei) and highly positive qRT-PCR remains to be determined. In this study, simultaneously performing IP-FISH and qRT-PCR upon CML patient samples, we assessed a simple method to characterize the overexpression of *BCR–ABL* rearrangement by gene deregulation. Using this method we identified CML patients with overexpression (OE) and resistance to imatinib treatment; moreover, this group of patients was associated with a worse overall survival.

Materials and methods

Patients

The samples for this study were peripheral blood (PB) mononuclear cells from 141 consenting CML patients (76 males and 65 females) treated with imatinib. Samples were studied if a patient failed to achieve any of the following: (1) complete hematological response by 3 months; (2) mayor cytogenetic response by 12 months. Patients were also included if they lost complete hematological or cytogenetic responses during chronic phase or progressed to an advanced phase of disease. Between one and three samples were investigated from each patient during the course of their disease. Mean disease duration before imatinib therapy was 26 (range 0–130) months, while the mean disease duration during imatinib treatment was 27 (range 3–68) months. Patients were classified according to disease phase: chronic 62%, accelerated 27%, and blastic 11%.

Evaluation of response and resistance mechanism

We estimated the number of genomic *BCR–ABL* copies by FISH, the expression level of *BCR–ABL* transcripts in blood leukocytes by qRT-PCR, and with the aim to determine the presence of point mutations, the *BCR–ABL* tyrosine kinase domain was sequenced using cDNA after RT-PCR amplification from total RNA.

Fluorescence *in situ* hybridization

For interphase-FISH, between 200 and 300 interphase nuclei were evaluated. FISH was performed using commercially available LSI BCR–ABL ES probe (Vysis, Downers Grove, IL, USA) according to the manufacturer's instructions. The signals were viewed with a Zeiss Axioskop (Zeiss, Heidelberg, Germany). Nuclei with a fused green and red or a yellow signal plus an extra signal in der(9) were interpreted as positive for the *BCR–ABL* translocation. The determined cut-off level for this probe in our laboratory was 1.5%.

Quantitative reverse transcriptase-PCR

Samples were processed as soon as possible after aspiration. Total leukocyte RNA was extracted from 10 mL of PB after lysis of red blood cells. RNA extraction was performed by TRIZOL (Invitrogen, Life technologies, CA, USA) method according to manufacturer's instructions. RNA was reverse transcribed using random hexamer primers and MMLV reverse transcriptase during 1 h at 37°C; cDNAs were stored at −20°C. Quantitative real-time PCR was performed using the LightCycler 2.0 instrument (Roche Diagnostics, Mannheim, Germany), based on the SYBR-Green method. Calculation of the transcript level expression was done by forming the ratio of the crossing points (Cp) of the transcript levels of *BCR–ABL* and the housekeeping gene *ABL*. Optimal reaction conditions for amplifying both *BCR–ABL* and *ABL* were as follows: 50

cycles of a 4 step PCR (95°C 10 s, 60°C 3 s, 72°C 12 s, 80°C 1 s) after an initial denaturation (95°C 10 min). The 20 μL qRT-PCR reaction mixtures contained 5 μL of sample cDNA, 1X PCR Mix (LC FastStart DNA Master SYBR Green I; Roche Diagnostics), 3.5 mM $MgCl_2$, 0.25 μM of each primer (*BCR/ABL*-forward 5'-TCC ACT GGC CAC AAA ATC ATA CAG T-3'; BCR/ABL-reverse 5'-TCA GAA GCT TCT CCC TGA CAT CCG T-3'; ABL-forward 5'-TGG AGA TAA CAC TCT AAG CAT AAC TAA AGG T-3'; ABL-reverse 5'-GAT GTA GTT GCT TGG GAC CCA-3'). qRT-PCR experiments were performed once, but if the *BCR–ABL/ABL* values were inconsistent with FISH result, the procedure was repeated. When the sensitivity was studied in serial 10-fold aqueous dilutions of cDNA (from 1 to 10^6), obtained by RT-PCR of RNA from K562 cells, a dilution of 10^5 was detectable when 1 μg of RNA was transcribed. Primer pairs efficiencies were also estimated from the given slopes in LightCycler software (Roche, Indianapolis, IN, USA); both primer pairs showed high and very similar real time-PCR efficiency rates (2.09 for *BCR–ABL* and 2.11 for ABL) in the investigated range $(1–10^5)$. For all qRT-PCR reactions, K562 cells were used as a positive control and lymphocyte cells from healthy donors as negative control. The reliability of qRT-PCR was tested performing repeated tests that could amplify *BCR–ABL* and *ABL* transcripts with an intra-assay coefficient of variation (CV) of 0.33% (*BCR–ABL*) and of 0.45% (*ABL*).

Kinase domain sequencing

The same cDNA samples used for qRT-PCR were employed to amplify and sequence a region corresponding to the ATP binding pocket and the activation loop of the kinase domain of *BCR–ABL*. A 1327-bp cDNA fragment was amplified by 10 PCR cycles with a 5' BCR-specific primer (5'-GAA GCT TCT CCC TGG CAT CCG T-3') and a 3' ABL-specific primer (5'-CCA GGC TCT CGG GTG CAG TCC-3'). Adding a third 5' ABL-specific primer (5'-GCG CAA CAA GCC CAC TGT CTA TGG-3') a second PCR round was carried out using an aliquot of the first PCR product, leading to a 579-bp fragment (5) that is finally sequenced, after column purification (Amersham Biosciences, Pittsburgh, PA, USA), using an ABI Prism 377 automatic DNA sequencer.

Statistical analysis

For categorical variables, frequencies, percentage, and distributions were summarized. For continuous variables, ranges, medians, mean, and standard errors were summarized. The means between pairs of continuous variables were analyzed by the *t*-test for paired differences. The correlation coefficients between pairs of continuous variables were calculated by Pearson correlation. In all analyses two-sided *P*-values of < 0.05 were regarded as significant. We used the area under the receiver operating characteristics (ROC) curve to summarize the ability of the test to discriminate an imatinib resistant patient for *BCR–ABL* overexpression from a responder patient or an imatinib resistant for other causes. The study is ongoing, but 30 July 2008, was the cutoff date for this analysis. We performed analyses of survival using the Kaplan–Meier method and differences between subgroups of patients receiving imatinib were calculated by the log-rank test. Data analyses were done with GRAPHPAD 4.0 statistical software (GraphPad Software, Inc, La Jolla, CA, USA).

Results

Real-time quantitative RT-PCR and FISH analysis

For this study 141 CML patients treated with imatinib were included. Eighty-eight patients were studied in chronic phase (CP), 38 in accelerated phase (AP) and 15 patients in blastic crisis (BC) (Table 1). We analyzed the results of all cases in which both FISH and qRT-PCR were applied concomitantly in the same patient's sample;

Table 1 Characteristics of the 141 patients with CML

	Chronic phase	Accelerated phase	Blast crisis	All subjects
	n = 88	*n* = 38	*n* = 15	*n* = 141
Gender				
Male	48	21	7	76
Female	40	17	8	65
Age (yr, mean)	50	49	41	49
Imatinib dose (*n*)				
400 mg	40	3	4	31
400–600 mg	29	13	2	26
>600 mg	19	22	9	36
Pre-imatinib (months, mean)	23	31	25	26
Imatinib treatment (months, mean)	32	31	18	27

	Chronic phase n = 74	Accelerated phase n = 34	Blast crisis n = 15	All subjects n = 123
Ratio BCR–ABL/ABL (mean % ± SE)	13.2 ± 2.7	36.3 ± 6.6	18.9 ± 9.1	20.3
FISH (mean % ± SE)	26.5 ± 3.4	54.8 ± 6.7	56.8 ± 14.1	38
BCR–ABL gene amplification (n)	3	2	2	7
Expression index (median)	0.228	0.337	0.086	0.288
ABL kinase point mutations (n)	10	18	4	32

Table 2 Results of the FISH and qRT-PCR analysis in 123 CML patients

taking in account that in 18 cases the BCR–ABL rearrangement was not detectable by both qRT-PCR and FISH, we decided to exclude these cases from this study (Table 2). The mean value (± Standard Error) of BCR–ABL/ABL ratio of the 123 remaining patients was calculated: for 74 CML patients in chronic phase was 13.2% ± 2.7, this value significantly increased ($P < 0.0001$) in accelerated phase (36.3% ± 6.6, $n = 34$) and blastic phase (18.9% ± 9.1, $n = 15$) of disease (Fig. 1). This figure shows also the patterns of molecular response (MR) according to the disease phase; MRs were calculated according to the Log reduction of the BCR–ABL/ABL level respect to a pool of eight patients (mean value 96.6%, baseline) studied at the time of diagnosis. Reduction was less than 1 Log in 56 patients (45.5%). It was ≥1 and <3 Logs in 56 patients (45.5%), and was equal to or more than 3 Logs (MaMR, Mayor Molecular Response) in 11 patients (9%). In all 123 patients between 200 and 300 interphase nuclei were evaluated for BCR–ABL rearrangement by FISH analysis. The results are expressed as the ratio (%) of Ph^+ nuclei to total nuclei. The mean FISH ratio was 26.5%

in CP, 54.8% in AP, and 56.8% in BC (Table 2). Gene amplification of BCR–ABL (when two or more signals of BCR–ABL rearrangements were detected by FISH) was observed only in 7/123 (5.7%) cases.

Comparison of real time PCR and FISH: expression index

In the 123 CML cases BCR–ABL/ABL mean ratio by qRT-PCR accounted for 11.41% ± 4.5 ($n = 27$) in the 0–5% FISH group, 13.7% ± 5.7 ($n = 45$) in the 6–35% FISH group, 27.2% ± 6.0 ($n = 37$) in the 36–85% FISH group and 40.7% ± 14.6 ($n = 14$) in the 86–100% FISH group (Fig. 2). The BCR–ABL/ABL calculated ratio showed a significant correlation with IP-FISH. Although the correlation is good (Spearman $r = 0.45$; $P < 0.0001$) a wide range in the BCR–ABL/ABL ratio was observed especially in patients who showed low percentage (<5%) of Ph^+ nuclei by FISH analysis. With the aim to determine overexpression of BCR–ABL transcript, we propose a method that correlates qRT-PCR and FISH data from the same peripheral blood sample. The ratio between BCR–ABL/ABL and IP-FISH data permit to calculate the EI for each patient; EI was estimated by calculating the ratio between real time PCR data (X_{qRT}) and FISH data (Y_{FISH}) (X_{qRT} / Y_{FISH}). This ratio allows estimating the rate of transcription per BCR–ABL fusion gene thus characterizing the OE.
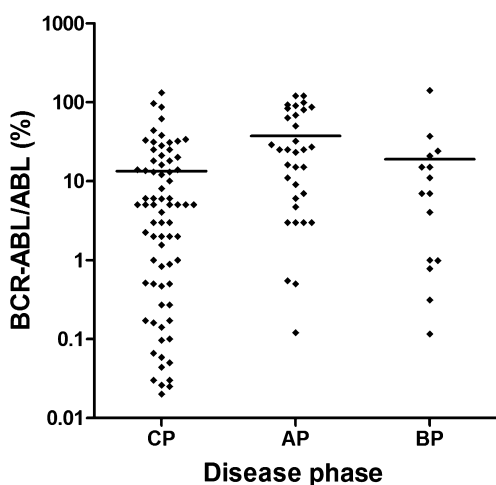

Figure 1 Real-time PCR quantification of BCR-ABL transcript in 141 chronic myeloid leukemia (CML) patients. The amount of BCR–ABL mRNA was expressed as a percentage ratio [(BCR–ABL/ABL) × 100]. Each point corresponds to the measurement for one patient. Eighty-eight patients were studied in chronic phase (CP), 38 in accelerated phase (AP) and 15 patients in blastic crisis (BC). Mean values for each group are indicated by the straight lines
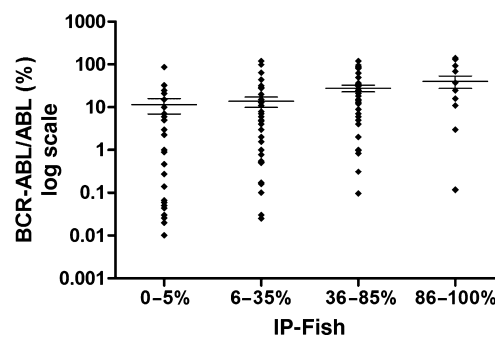

Figure 2 Comparison of the BCR–ABL levels derived from qRT-PCR vs. fluorescence in situ hybridization (FISH) analyses. Quantitative comparison of the BCR–ABL/ABL ratio as determined by qRT-PCR and four FISH groups classified according to the percentage of Ph^+ nuclei as determined by IP-FISH. The lines indicate the mean with standard error.
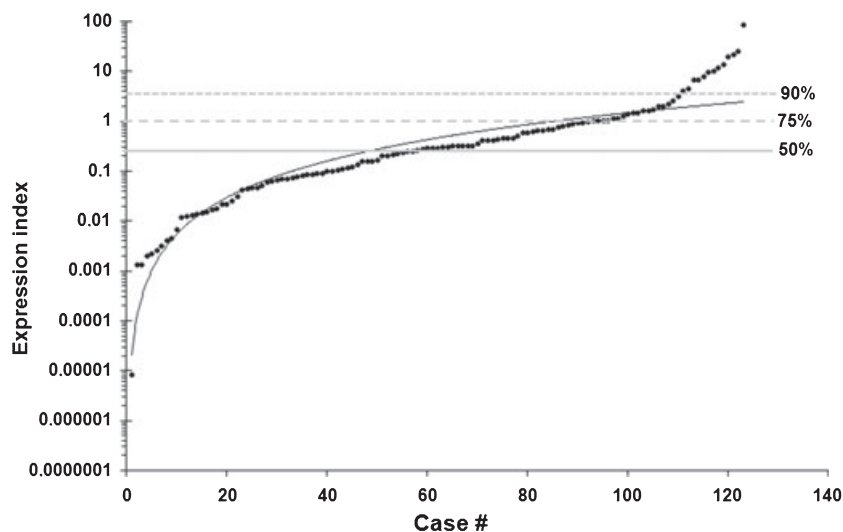
Figure 3 Expression Index graph. Each point corresponds to the calculated expression index (EI) for one patient. Straight line at percentile 50 represents the median value of EIs (0.288); the middle dot line indicates the percentile 75 of EIs (0.984). Finally the upper dot line represents the percentile 90 of EIs (3.825) including 13 patients that showed more than a 10-fold increase in the rate of *BCR–ABL* transcription.

ABL is the most widely used reference gene for *BCR–ABL* quantitation. Taking into account the primers design, *BCR–ABL* transcript provides cDNA template for both *BCR–ABL* and *ABL* PCR amplification. This may lead to an underestimation of the BCR–ABL/ABL ratio. Therefore, we calculated a second expression index (named corrected expression index, cEI) with the aim to quantify the impact of the spurious contribution (the rationale for cEI calculation is shown in Fig. S1). Statistical comparison and correlation between EI and cEI in our population indicate no significant differences in both the curve shapes and the neat discrimination of the overexpressing *BCR–ABL* group (Fig. S2). Therefore, for simplicity, EI was chosen as a measure to detect *BCR–ABL* overexpression.

The EIs were determined in all 123 CML patients obtaining a median EI (M_{EI}) value of 0.288 (Fig. 3). Percentile analysis was performed to stratify the patients in different groups; those cases included in percentile 90 showed an increment of EI above 1 Log respect to the median value. Thirteen cases were included in the percentile 90 that we defined as cases with overexpression of *BCR–ABL* transcripts (13/123). The 123 samples were also used to quantitatively compare EIs and IP-FISH; FISH groups were differentiated using four different cutoff levels as shown in Fig. 4, which shows that EIs values are very stable in the two groups with high percentage of FISH while quite variable in the lower FISH groups.

Receiver operating characteristics analyses

We also evaluated the EIs using ROC curve, which permit an analysis of the tradeoff between sensitivity
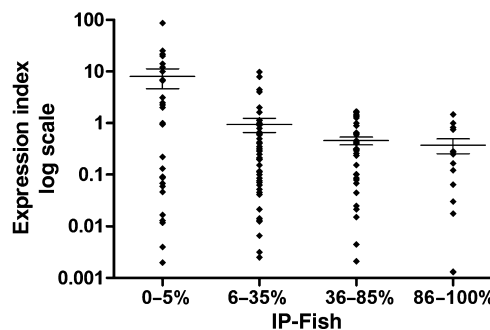

Figure 4 Comparison of the expression indices (EIs) vs. fluorescence *in situ* hybridization (FISH) analyses. Quantitative comparison of the rate of *BCR–ABL* transcription (EI) and four FISH groups classified according to the percentage of Ph+ nuclei as determined by IP-FISH. The lines indicate the mean with standard error.

and specificity at variable cut points. The ROC curve was constructed using EI values from two groups of CML patients defined as: test group ($n = 25$) with discordant data (FISH < 5%, MR > 0.01%) and control group ($n = 95$) with concordant data including responding patients (FISH < 5%, MR < 0.01%) and not responding patients (FISH > 5%, MR > 0.01%). The area under the ROC curve (AUC) quantifies the overall ability of the test to discriminate between those individuals with or without *BCR–ABL* overexpression. We obtained a significant ($P < 0.01$) AUC value of 0.68 (99% CI, 0.49–0.87). In order to maximize the sensitivity we chose an EI cutoff of 1.836 obtaining a sensitivity of 95% (95% CI, 87.10–99.04%) and a specificity of 61% (95% CI, 35.75–82.70%). This cutoff is lower than the value we obtained by the percentile stratification (2.880); therefore, more patients (17/123,

5

Table 3 Characteristics of the 17 patients with BCR-ABL overexpression (ranked by EI)

Code	FISH	qPCR	EI	Log EI[1]	Phase disease	BMT[2]	Gene amplification	Point mutation	Later point mutation
3053	1	86	86	2.5	AP	no	no	no	no
3546	1	25	25	1.9	CP	no	no	no	no
3033	4	86.7	21.7	1.9	CP	no	no	no	no
3412	0.5	10	20	1.8	CP	no	no	no	no
3529	0.5	7	14	1.7	BP	no	no	no	no
3180	0.5	6	12	1.6	CP	no	no	no	M351T
3034	0.5	5	10.1	1.5	CP	no	no	no	no
3252	10	98	9.8	1.5	AP	no	no	V289F	no
3050	8	63.2	7.9	1.4	AP	no	no	no	no
3073	3	20.7	6.9	1.4	BP	no	no	no	no
3927	5	33	6.6	1.4	CP	no	no	M351T	no
3022	10	43.8	4.4	1.2	CP	no	no	no	no
3193	30	120	4	1.1	AP	no	no	no	E355G
3129	4.8	15	3.1	1.0	BP	no	no	no	no
3038	2	5	2.5	0.9	CP	yes	no	no	no
3622	10	20	2	0.8	CP	no	no	no	no
3484	0.5	1	2	0.8	CP	no	no	no	no

[1]Log fold change with respect to the median EI.
[2]BMT (Bone Marrow Transplantation).

13.8%) are included in the overexpressing group (Table 3).

Patient's samples: kinase domain mutation analysis

Point mutations in the ABL tyrosine kinase domain were detected in 32 out of 123 patients (26%) at the time of resistance. Throughout this analysis we could observe that only two patients with point mutations in the kinase domain, belonged to the overexpression group (Tables 2 and 3). Notably, both aminoacid changes (M351T and V289F) are reported as imatinib sensible variants, in agree with the fact that it is improbable that two different resistance mechanisms could occur in the same patient.

Survival analysis

We analyzed the effect of overexpression on survival from either the time of diagnosis or the onset of therapy with imatinib. After a median follow-up from diagnosis of 59 months (range, 3–218 months), seven of 16 (44%) patients with overexpression, nine of 27 (33%) patients with mutation and/or amplification and 15 of 76 (20%) patients without the above features ('wild-type') died ($P < 0.05$). Comparing OE survival curve with 'wild-type' curve the Hazard ratio is 2.9 (95% CI of ratio 1.4–16.1) indicating that the rate of death for subjects with overexpression is threefold the rate of the 'wild-type' patients (Fig. 5A). Moreover, patients have been followed for a median of 46 months (range, 2–91 months) from the start of therapy with imatinib; when OE
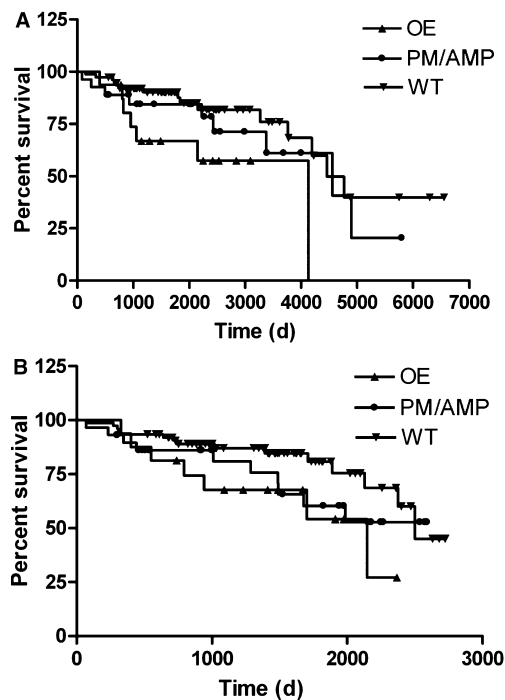

Figure 5 Overall survival by overexpression status from the time of diagnosis (A) and the onset of therapy with imatinib (B). Patients who are found to have an EI higher than the cutoff established in our laboratory, are considered as overexpressing (OE) patients and grouped together. PM/AMP, patients with kinase domain point mutation or gene amplification; WT, 'wild type' patients.

survival curve was compared with 'wild-type' curve (Fig. 5B), the development of overexpression was associated with a significantly shorter survival ($P < 0.05$).

Together, these findings suggest that overexpression confer a worse survival.

Discussion

As the presence of the *BCR–ABL* gene and its product is considered a surrogate for disease activity, its disappearance is regarded as the prerequisite for cure and the ultimate therapeutic goal. Different methods are available to determine the presence of *BCR–ABL*-positive cells in CML: FISH determines the proportion of Ph$^+$ cells at the DNA level, while real-time PCR measures BCR-ABL transcripts on the mRNA level. In this study, we report the development of a method that, combining both techniques, is useful to quantify the EI of the leukemic cell clone for detection of *BCR–ABL* transcript overexpression. This simple calculation improves diagnosis and monitoring of all CML patients under treatment with imatinib.

There are few publications where *BCR–ABL* rearrangement is simultaneously studied by quantitative PCR, FISH, and by kinase domain sequencing. In accordance with results reported by Kim *et al.* (15), which observed that in 67% of CML cases with high tumor burden FISH and qRT-PCR had concordant results, we also found a good correlation between the two assays. On the contrary, though in a small number of samples, Chomel *et al.* (16) reported no significant relationship between the percentage of positive nuclei by FISH and qRT-PCR, suggesting that the *BCR–ABL* can persist unexpressed in non-proliferating cells. Moreover, while studying mechanisms of resistance to imatinib therapy, Hochhaus *et al.* (8) reported 6% of cases with genomic amplification as determined by FISH, and 13% of cases with overexpression, considering overexpressed those cases where at least a 10-fold increment was observed in the ratio of *BCR–ABL*/G6PD, comparing measurements prior to treatment and at the time of resistance. However, the raise observed by these authors may be due to an increase of the abnormal clone without a real overexpression of *BCR–ABL* gene. Tabone *et al.* (17) studying another tyrosine kinase receptor, published that gene amplification of c-Kit in gastrointestinal stromal tumors (GIST) is not implicated in Kit overexpression in the majority of GISTs, suggesting up-regulation of c-KIT gene transcription on both mutated or wild type forms. Resistance to imatinib treatment in CML is mainly associated to three *BCR–ABL* kinase dependent mechanisms: acquired mutations in the kinase domain of *BCR–ABL* protein, gene amplification at genomic level or transcript overexpression. The amplification of the *BCR–ABL* gene in imatinib-resistant leukemic cells was initially described in a LAMA84R cell line. FISH analysis in this cell line showed that the mechanism causing *BCR–ABL* protein overexpression was the presence of about 15 copies of the *BCR–ABL* gene (7). This mechanism has also been observed in clinical samples from patients, who are resistant to imatinib with a reported overall frequency of about 18% (18). However, in some cases *BCR–ABL* is overexpressed in lack of gene amplification (19) indicating the existence of other mechanisms such as changes in the transcriptional control of *BCR–ABL* (20). In a few words overexpression may be due to the presence of numerous copies of *BCR–ABL* rearrangement within the same cell, or to an enhanced transcription rate of a unique oncogenic sequence. In order to characterize the latter mechanism, we decided to perform FISH and qRT-PCR in the same peripheral blood sample of 123 CML cases selected for their clinical resistance to imatinib.

Interestingly, taking into account the percentage of positive interphase nuclei as estimated by FISH analysis, we could observe that in a fraction of patients the relative quantification of *BCR–ABL* by real time PCR significantly increased with respect to the median transcription rate of *BCR–ABL*. This finding suggests that in some cases the transcription rate of *BCR–ABL* is higher than normally expected. Therefore, we decided to quantify this transcription rate for each CML sample, and report it as the EI, i.e. the ratio between qRT-PCR and FISH both measured in the same PB sample. The EIs achieved in all cases allowed us to estimate the median value (0.288) of *BCR–ABL* transcript expressed by a tumor cell. Using ROC analysis we could determine 1.836 as a faith cutoff value to select patients ($n = 17$) with overexpression. The 17 cases are resistant to the treatment with imatinib, since they show a *BCR–ABL* reduction lower or equal to 2 Logs with respect to the baseline value; moreover, in nine cases an acceleration of the disease is observed. Noteworthy, patients belonging to the overexpressed group showed a steady overexpression status over repeated quantification; this is very important to discard the possibility that the observations were sporadic. Interestingly, in two cases (see Table 3) a mutation in the kinase domain of the protein (not yet detectable at the time of the expression study) emerged several months later, suggesting that the overexpression condition would not preclude the development of the mutation. On the other hand, almost all patients showing a point mutation in the kinase domain, presented both a high number of *BCR–ABL* transcripts and Ph$^+$ nuclei (no overexpression, EIs close to 0.288), suggesting that generally when the leukemic cell clone is mutated the transcription rate of *BCR–ABL* is not significantly increased.

Moreover, when the presence of overexpression is analyzed as if it was a molecular characteristic and patients grouped together, age, duration of the disease, prior

IFN-α, and advanced stage at the time of studying were not correlated with the occurrence of overexpression. Importantly, we observed that this molecular characteristic influences the overall survival, with earlier deaths for patients with overexpression.

In conclusion, we defined an EI for each patient, which permits to quantify the intensity of *BCR–ABL* transcription rate by tumor cell. In this way it is possible to identify whether the overexpression is due to deregulation of *BCR–ABL* transcription. The detection of high *BCR–ABL* transcription rate may be considered as overexpression and a potential indicator of resistance to treatment with imatinib. For these cases resistance would depend on a particularly hostile clone, due to the high transcription rate of tumor cells. Since these cases showed to die earlier, EI measuring may be useful to predict a group of patients with poorer survival; however, this kind of analysis remains mainly exploratory at this time, and further prospective studies are necessary to prove its predictive value. Whether this variation in *BCR–ABL* transcription rate can be overcome with dose escalation or second-generation TK inhibitors has to be determined *in vitro* using primary CML cells from overexpressing patients. Also, monitoring of imatinib plasma levels could be very useful for the management of CML patients with high EI. It would be interesting to determine whether a threshold that is correlated with the presence of overexpressing clones exists. Finally, based on the experimental findings reported by Tipping and coworkers (21), it could be speculated that selected patients who become refractory to imatinib for overexpression may benefit from a second course of therapy after an interval of this inhibitor.

Acknowledgements

Supporting Information

Additional Supporting Information may be found in the online version of this article:
Figure S1. Graphical representation and rationale of *BCR–ABL* gene expression indexes.
Figure S2. Comparison of EI and cEI expression indexes.

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