original paper

haematologica 2001; 86:167-173
http://www.haematologica.it/2001_0167.htm

Competitive polymerase chain reaction as a method to detect the amplification of *bcr-abl* gene of chronic myeloid leukemia

FABIO CAMPANINI,* MARIA ALESSANDRA SANTUCCI,° LAURA PATTACINI,* GIANLUCA BRUSA,* MILENA PICCIOLI,* ENZA BARBIERI,# LUCIO BABINI,# SANTE TURA*

*Istituto di Ematologia e Oncologia Medica "L.A. Seràgnoli", °Dipartimento di Patologia Sperimentale, #Istituto di Radioterapia "L. Galvani", University of Bologna Medical School, Bologna, Italy

Correspondence: Fabio Campanini, M.D., Istituto di Ematologia e Oncologia Medica "L.A. Seràgnoli", via Massarenti, 9, Ospedale S. Orsola, Università degli Studi di Bologna, 40138 Bologna, Italy. Phone: international +39-051-6363680 ext. 4036 – Fax: international + 39-051-6364037 - E-mail: campa9@inwind.it

Background and Objectives. The chimeric product of the *bcr-abl* rearranged gene is critical in the pathogenesis of chronic myeloid leukemia (CML), yet its role in the progression of the disease remains unclear. There is some evidence that increased *bcr-abl* expression levels, possibly due to gene amplification, precede the clonal evolution of CML hematopoietic progenitors toward a fully transformed phenotype and might be involved in their resistance to interferon-alpha or tyrosine kinase inhibitors.

Design and Methods. To quantify the *bcr-abl* gene both at the genomic and at the transcriptional levels we developed a competitive polymerase chain reaction (PCR) strategy. The competitive PCR technique is based upon the co-amplification of the sample template (target) together with increasing amounts of a DNA fragment (competitor) sharing with the target the primer recognition sites, but differing in size. We constructed a competitor for the quantification of both b_2a_2 and b_3a_2 alternative splicing forms of the *bcr-abl* chimera and established the accuracy and reproducibility of our competitive strategy in a clone of the murine 32DG hematopoietic cell line (32D LG7), which bears a stable integration of a single copy of p210 *bcr-abl* fusion gene. We utilized this technique to follow, over a period of 200 days, the fusion gene copy numbers and transcription rates in several p210 *bcr-abl*-transduced 32D cell clones, an experimental condition mimicking the evolution of CML myeloid progenitors *in vivo*.

Results. Our results are consistent with p210 *bcr-abl* overexpression but not gene amplification associated with their clonal evolution. Increased p210 *bcr-abl* transcription rate is associated with the abrogation of radiation-induced apoptotic cell death, suggesting a role for the chimeric gene expression level in cell life expectancy after a genotoxic insult.

Interpretation and Conclusions. We conclude that the assessment of gene amplification and expression might serve to improve prognostic classification and follow-up of CML patients.

Key words: chronic myeloid leukemia, p210 *bcr-abl*, gene amplification

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder of the primitive hematopoietic stem cell that progresses in distinct clinical stages. The initial indolent phase, the chronic phase, may last for years and is characterized by the illegitimate expansion of a clonal myelopoiesis, resulting in the elevation of peripheral blood white cells (and sometimes platelets) which still retain the capacity to differentiate into normal and functional granulocytes.[1] Ultimately, the chronic phase progresses into the terminal stage, named blast crisis, which leads to the accumulation of primitive progenitor cells arrested at an early stage of either myeloid or lymphoid differentiation.[2] The cytogenetic hallmark of CML is the Philadelphia chromosome (Ph1),[3] a balanced reciprocal translocation resulting in the aberrant transfer of the *c-abl* proto-oncogene from chromosome 9 to a site adjoining the breakpoint cluster region (*bcr*) of the BCR gene on chromosome 22.[4] The chimeric *bcr-abl* gene transcribes a 8.5 kb hybrid mRNA, that translates a 210 kDa protein ($p210^{bcr\text{-}abl}$), endowed with constitutively active protein tyrosine kinase activity.[5,6] $p210^{bcr\text{-}abl}$ is crucial in the pathogenesis of CML, but is probably not fundamental for the progression of the disease.[7] That the p210 product of *bcr-abl* fusion gene has, indeed, a critical role in the pathogenesis of CML has been proved by its transforming potential on hematopoietic progenitor cells *in vitro* and by the development of a CML-like hematopoietic neoplasm in lethally irradiated mice whose hematopoiesis was reconstituted with *bcr-abl*-transfected bone marrow cells.[8,9] However, its role in the progression of the disease is still obscure. So far, there is evidence that increased *bcr-abl* expression levels, possibly due to gene amplification, precede the clonal evolution of CML hematopoietic progenitors toward a fully transformed phenotype[10] and are likely involved in the cells' resistance to interferon-α or tyrosine kinase inhibitors.[11]

The quantification of *bcr-abl* fusion gene at the DNA level (quantitative Southern blot or fluorescence *in situ* hybridization) (FISH)[12] or at the RNA level (quantitative

reverse transcription-polymerase chain reaction)[13] has been attempted for the clinical purpose of monitoring residual disease following allogeneic bone marrow transplantation or during treatment with interferon-α or the tyrosine kinase inhibitor, STI571. Far fewer studies have addressed the matter of significance of the aberrant gene expression levels in the progression of associated disease and, up to date, there is no clear evidence about the impact of $p210^{bcr\text{-}abl}$ in the prognostic evaluation of CML patients at diagnosis.

In the study presented here we describe a competitive polymerase chain reaction (PCR) strategy that we developed in order to quantify $p210^{bcr\text{-}abl}$ expression both at genomic and transcriptional levels. To this purpose, we performed sequential measures of *bcr-abl* gene copy number and transcript molecule in several 32DG cell clones stably transducing the $p210^{bcr\text{-}abl}$ fusion gene, continuously maintained in culture. This experimental condition appears to mimic the clonal evolution of CML hematopoietic progenitor cells *in vivo*. Our results failed to find any correlation between the chimeric gene copy number and its transcriptional expression level, supporting the hypothesis that gene amplification in CML may occur through multiple mechanisms. In conclusion, our strategy may help to define the biomolecular steps involved in disease progression possibly important in improving the prognostic classification of CML patients.

Design and Methods

Cell lines and culture conditions

32D cell line is a murine myeloid progenitor cell line originally isolated from the non-adherent fraction of long-term bone marrow cultures.[14] The cell clone denominated 32DG was obtained from G. Rovera (The Wistar Institute, Philadelphia, PA, USA) and is strictly dependent on interleukin 3 (IL-3) for growth. We used electroporation to introduce into the 32DG cell line a full length $p210^{bcr\text{-}abl}$ construct subcloned into the pGD retroviral vector under the control of LTR promoter of the myeloproliferative sarcoma virus along with a neomycin phosphotransferase gene allowing for selection in the neomycin analog G418. The construct was kindly donated by N. Carlesso (Massachusetts General Hospital, Boston, MA, USA), and a more detailed description of its construction has been given in a published paper.[15] Electroporation was performed at 0.25V/960mF (Equibio Easyject, Optima). Cells were allowed to recover for 48 hours in RPMI with 10% FCS (Gibco), in the presence of 10% of Wehi-3 conditioned medium as the source of IL-3. Cells expressing the electroporated construct were then selected in medium to which 1 mg/mL of G418 had been added. After the initial selection, individual subclones were obtained by cloning in semisolid medium (methylcellulose) in the absence of IL-3. The 32DG LG7 cell clone stably expressing the $p210^{bcr\text{-}abl}$ fusion gene was kindly donated by P. Laneuville (Royal Victoria Hospital, Montreal, Quebec, Canada). It was generated by transfection of a $p210^{bcr\text{-}abl}$ construct under the control of the same promoter.[16] All $p210^{bcr\text{-}abl}$-transduced 32D cell clones were maintained in RPMI culture medium (Gibco) with 10% FCS (Gibco), 1% L-glutamine, 1% sodium pyruvate and antibiotics. Methylcellulose cell cultures were performed in 0.9% methylcellulose (Sigma) in IMDM (Gibco) with 30% FCS. Colonies (aggregates containing >50 cells) were either scored or picked for cloning after 7-10 days incubation in 5% CO_2 in air at 37°C.

Genomic DNA and RNA isolation

Total genomic DNA and RNA were isolated with DNeasy and RNeasy Tissue kits (Qiagen, Germany), respectively, and according to the manufacturer's instructions. The exact amount of both nucleic acids extracted was measured by optical density at 260 nm and the integrity of the nucleic acids was assessed by electrophoresis on agarose gel.

Reverse transcription and competitive PCR

Reverse transcription (RT) was performed with 2 μg of total RNA in 50 μL of reaction mixture containing 1xRT buffer (50 mM Tris HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$) 4 μM DDT (Life Technologies), 0.8 mM dNTPs (Life Technologies), 200 U M-MLV reverse transcriptase (Life Technologies), 40 U RNAse inhibitor (Boehringer Mannheim) and 1.5 μM random hexamers (Perkin Elmer). The RT reaction was carried at 37°C for 1 h, followed by 5 min at 95°C to inactivate the enzyme. PCR reactions were performed with 2 μL of RT products in 30 μL of PCR reaction mixture (50 nM Tris pH 8.4, 250 μg/mL BSA, 3 mM $MgCl_2$, 2 mM dNTPs and 4 U Taq polymerase KlenTaq from Bionova, Italy) added to 0.5 μM specific primers (CHS3 and CHS2 for *bcr-abl* gene and CHS7 and CHS6 for the murine glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene as internal control). Genomic DNA PCR reactions were performed with 100 ng of purified genomic DNA in 30 μL of reaction mixture to which specific primers had been added (Table 1). Competitive PCR reactions were carried by addition of scalar amounts of specific competitor DNA fragments to a fixed quantity of the cDNA (2 μL) or of the genomic DNA (100 ng), followed by PCR amplification using the respective primer pair. The PCR reactions were performed in microcapillary tubes using a Rapid Cycle DNA Amplification instrument (Idaho Technology), according to the following cycle profile: denaturation at 98°C for 0'', annealing at 59°C for 0'' and extension at 72°C for 10''. Thirty cycles were carried out for PCR amplification of *bcr-abl* cDNA, G3PDH cDNA and gene. Thirty-five cycles were carried out for PCR amplification of genomic *bcr-abl*.

After amplification, PCR products were resolved by gel electrophoresis, stained by ethidium bromide and quantified by densitometry. According to the principles of competitive PCR, the ratio between the amplification products is linearly correlated to the input amount of competitor in the reaction.[17] Thus the equivalence point (i.e. the amount of competitor resulting in a 1:1 ratio between the two products) was calculated according to

Table 1. Sequence of primers used for reverse transcription and PCR amplification.

Gene[ref]	*Oligonucleotide name*	*Sequence 5'->3')*	*cDNA position*	*Fragment size Template*	*Fragment size Competitor*
G3PDH[24]	CHS6	TCATCATCTCTGCCCCCTCTG	416-436	440	392
	CHS7	CGCCTGCTTCACCACCTTCTT	855-836		
bcr-abl[28]	CHS3	AGCTTCTCCCTGGCATCCGTGGA	418-440 (bcr)	381 bp*	343
	CHS2	CCCATTGTGATTATAGCCTAAGA			

**Corresponding to b3a2 transcript.*

the equation of the line fitting the experimental points. Due to the size difference between competitor and sample templates (38 bp for *bcr-abl* and 48 bp for G3PDH) the number of competitor molecules at the equivalence point was multiplied by 0.9 for *bcr-abl* and 0.89 for G3PDH (these values correspond to the ratio in size of competitors and sample template PCR products). These numbers represented the absolute numbers of single gene molecules per microgram of total RNA or per 100 ng of total genomic DNA. Each quantification was repeated three times in at least three separate experiments for each cell clone.

Construction of competitor templates

A competitor for quantification of both b_2a_2 and b_3a_2 alternative splicing forms of *bcr-abl* chimera was constructed as follows. The PCR product obtained from the b_2a_2 rearranged sequence was cloned into the vector pGEMEX-T (Promega) according to the manufacturer's instructions and by standard cloning techniques.[18] This construct, named pMB75, was then cut within the b_2a_2 sequence by restriction with Bsu361 and the generated DNA ends were filled-in using Klenow DNA polymerase. A 37 bp DNA fragment present in a commercially available molecular weight marker preparation (Boehringer Mannheim, DNA molecular weight marker VIII) was separated and purified from other bands through a polyacrylamide gel, filled-in and ligated with the linearized pMB75 vector. The plasmid obtained (pMBc11) was purified, quantified and used for competitive PCR experiments. pMBc11 competitor plasmid is 3.65 Kbp in size and thereby 1 ng equals 2.5×10^8 molecules.

Plasmid pMBc12, used for the competitive PCR quantifications of murine G3PDH cDNA, contains a 58 bp deletion within the PCR primer recognition sites. It was constructed by an application of the recombinant PCR methodology[19] using a primer carrying a region of homology to an internal portion of G3PDH cDNA at the 3' end. The recombinant PCR product was cloned in the vector GEMEX-T and was called pMBc12. It was 4.06 Kbp in size and thereby 1 ng equals 2.25×10^8 molecules.

Western blot analysis

Immunoblotting was performed according to standard techniques.[15] Briefly, 2×10^7 cells, washed twice in cold PBS, were lysed in 200 µL of lysis buffer (10 mM Tris pH 8, 150 mM NaCl, 10 nM iodacetamide, 1% chaps, 0.02% sodium azide, with protease inhibitors trypsin, leupeptin, sodium ortovanadate and phenylmethylsulfonylfluoride). Equal protein amounts, measured by the Bradford assay, were boiled 5 min in 2× SDS sample buffer and analyzed by SDS electrophoresis on 10% polyacrylamide gels. Following electrophoresis, gels were electrophoretically transferred onto nitrocellulose (Schleicher and Schuell). Immunoblots with anti-abl antibody K-12 (Santa Cruz) were performed by diluting the antibody 1:2000 in TBS with 0.01% Tween 20 and visualized by enhanced chemioluminescence detection (ECL) (Amersham Corp.).

Apoptosis

Apoptotic cell death was assessed in parental and $p210^{bcr\text{-}abl}$-transduced 32DG cell clones 16 h after low dose (4 Gy), low dose rate (0.05 Gy/min) γ irradiation. Briefly, cells in exponential growth phase were irradiated under a cobalt unit with the source calibrated in order to deliver the above dose rate at the flask surface. After irradiation, cells were allowed 16h at 37°C in which to recover or die. Thereafter, DNA was extracted as described in a previous section and resolved by electrophoresis through 1% agar. Apoptotic cell death resulted in DNA fragmentation, visualized under UV light following staining with ethidium bromide.

Results

The aim of our study was that of assessing whether gene amplification, possibly occurring either at genomic or transcriptional level, might represent a biomolecular event associated with clonal evolution of CML progenitor cells. To this purpose we followed *bcr-abl* gene copy number and transcription rate in seventeen $p210^{bcr\ abl}$-transduced 32D cell clones by means of a competitive PCR stra- tegy. The $p210^{bcr\text{-}abl}$-transduced 32D cell clones were generated by electroporation into the 32D cell line named G[20] of a pDG vector containing the $p210^{bcr\text{-}abl}$ (a_2b_2) cDNA under the control of the myeloproliferative sarcoma virus LTR promoter along with the neomycin resistance gene, selected in medium to which the neomycin-analog G418 had been added and cloned in semisolid growth medium (methylcellulose). All of them were preliminary assayed for the presence, transcription and translation of *bcr-abl* chimera by means of FISH, RT-PCR, immunostaining and Western blot analysis (data not shown and Figure 1). In particular, FISH analysis revealed

Figure 1. Protein expression levels of p210 bcr-abl were assessed by immunoblotting in the 32D LG7 cell clone (lane 1) and p210 bcr-abl transduced 32DG cell clones B (lanes 2-8). Equal amounts of protein lysates (100 mg/lane), properly processed as described in the *Design and Methods* section were electrophoretically separated, blotted and hybridized with anti-abl antibody K-12 (Santa Cruz). Lanes 1, 2, 3, 4, 5, 6, 7, 8: 32DG LG7, 1B, 3B, 4B, 5B, 6B, 7B, 8B cell clones, respectively.

the presence of multiple copies of the gene in virtually all p210$^{bcr\text{-}abl}$-transduced 32D cell clones, although it was impossible to substantiate their number and integration sites, likely due to the too small construct size. Our result is consistent with a previous study indicating the difficulty in succeeding in the assessment of gene integration by fluorescent probes in bcr-abl-transduced murine cell lines.[21] The competitive PCR technique is based upon co-amplification of the sample template (target) together with increasing amounts of DNA fragment (competitor) sharing with the target the primer recognition sites, but differing in size. According to the principles of competitive PCR, when the ratio between the amount of amplification products is plotted against the amount of competitor added, the experimental points are fitted by a straight line.[22,23] Accordingly, the number of molecules of competitor corresponding to a 1:1 ratio, corrected by the difference in size between competitor and sample templates, is equivalent to the number of molecules of input RNA in the sample. In preliminary experiments we used titration assays to establish the accuracy and reproducibility of our competitive strategy, related to the murine glyceraldehyde-3-phosphate deydrogenase (G3PDH) housekeeping gene whose level was kept as indicative of the amount of amplified genomic DNA and cDNA (Figure 2).[24]

In our study we assumed as equal to 1 the number of molecules assessed by competitive PCR on genomic DNA and cDNA (respectively 2.8×10^4/100 ng and 2.25×10^6/µg) of the 32D LG7 cell clone, which bears one single *bcr-abl* integration site/cell.[16] The *bcr-abl* copy number/100 ng of genomic DNA showed very little heterogeneity in all seventeen *bcr-abl*-transduced 32D cell clones assayed 60 days after electroporation: it ranged between 4 and 6, compared to the 32D LG7 control value (Figure 3A). It did not undergo any later increase in seven p210$^{bcr\text{-}abl}$-transduced 32D cell clones denominated B assayed 90, 120 and 200 days after electroporation (Figure 3B). Conversely, the fusion gene transcription rates exhibited much greater heterogeneity both in an early phase following electroporation (60 days), when the *bcr-abl* mRNA molecule number was increased by 7.6-20.8 fold compared to

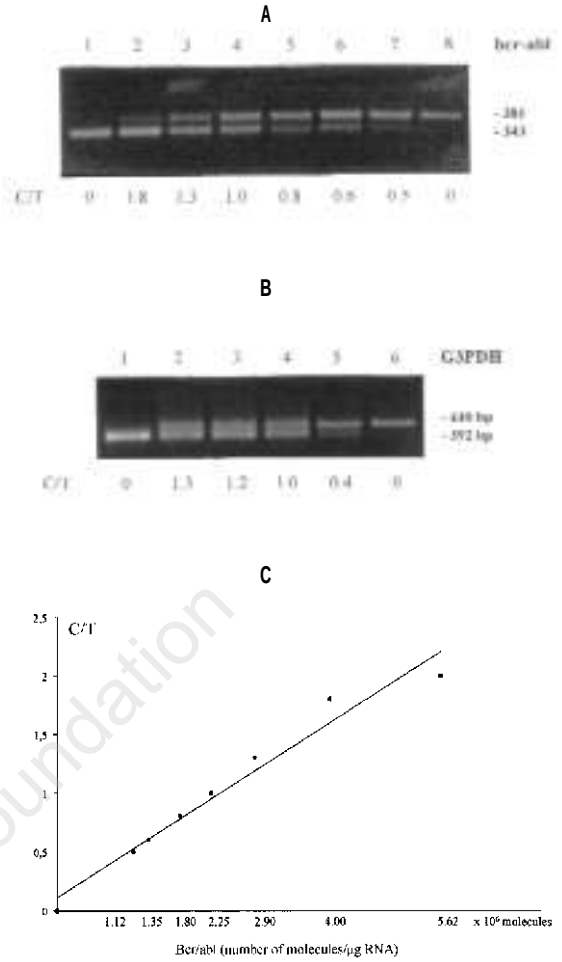

Figure 2. Example of competitive PCR relative to bcr-abl (A) and G3PDH (B) in p210$^{bcr\text{-}abl}$-transduced 32D LG7. For each amplification of *bcr-abl*, the ratio between the intensities of the bands (evaluated by densitometric scanning) was plotted against the amount of competitor added (C). The amounts of competitor for bcr-abl were 25 pg (lane 1), 18 pg (lane 2), 13 pg (lane 3), 10 pg (lane 4), 8 pg (lane 5), 6 pg (lane 6), 5 pg (lane 7) and 0 pg (lane 8), corresponding to 5.62×10^6, 4×10^6, 2.9×10^6, 2.25×10^6, 1.8×10^6, 1.35×10^6, 1.12×10^6 and 0 molecules, respectively. The amounts of competitor for G3PDH were 10 pg (lane 1), 7.8 pg (lane 2), 7.2 pg (lane 3), 6 pg (lane 4), 2.4 pg (lane 5) and 0 pg (lane 6), corresponding to 2.25×10^7, 1.75×10^7, 1.62×10^7, 1.35×10^7, 5.4×10^6 and 0 molecules, respectively. The expression level of the housekeeping G3PDH gene did not show any significant variation in p210$^{bcr\text{-}abl}$ transduced 32DG clones under any of the experimental conditions (range 9.6×10^6/1.5×10^7 molecules/µg total RNA). *T: template; C: competitor C/T: competitor/template ratio.*

the 32D LG7 control value (Figure 3A), and after longer intervals (90, 120 and 200 days), when it was further increased by 28.4-42.5 fold (Figure 3B). The expression level of the G3PDH housekeeping gene did not undergo significant change at any time after electroporation in

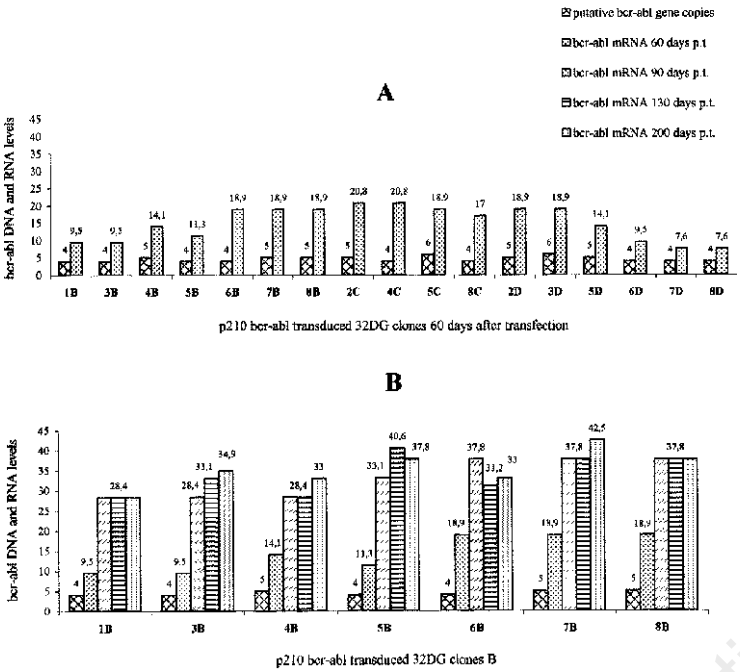

Figure 3. *Bcr-abl* gene copy number and RNA levels in seventeen p210$^{bcr-abl}$-transduced 32DG cell clones 60 days after electroporation (A). Clones denominated B were followed for 90, 130 and 200 days after electroporation (B). We referred to genomic and retrotranscribed DNA of 32D LG7 cell clone as the unit for the gene copy number and the transcription rate, since it has been proved to harbor a single integrated copy.[16] The increases of *bcr-abl* gene copy number / 100 ng of genomic DNA and mRNA molecules / μg total RNA are therefore compared to the control value of 32D LG7 chimeric gene and mRNA levels considered equal to 1.

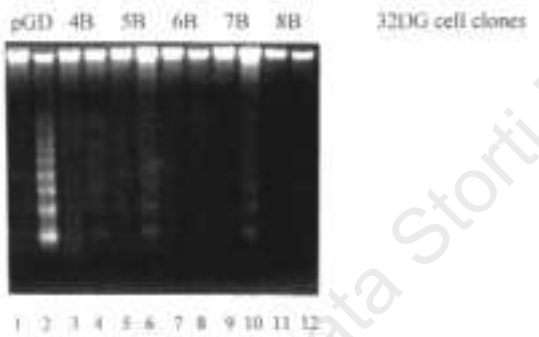

Figure 4. Fragmentation assay. Apoptotic cell death was assessed in 32DG transfected with pGD vector alone as a control and p210$^{bcr-abl}$-transduced 32DG cell clones B 16 h after low dose (4 Gy), low dose rate (0.05 Gy / min) γ irradiation. After irradiation, cells were allowed 16 h at 37°C in which to recover or die. Thereafter, DNA was extracted as described in *Design and Methods* and resolved by electrophoresis through 1% agar. Apoptotic cell death resulted in DNA fragmentation, visualized under UV light following staining with ethidium bromide. Lanes 1, 3, 5, 7, 9, 11: pGD-32DG, 4B, 5B, 6B, 7B, 8B non-irradiated cell clones, respectively. Lanes 2, 4, 6, 8, 10, 12: pGD-32DG, 4B, 5B, 6B, 7B, 8B irradiated cell clones, respectively.

any of the p210$^{bcr-abl}$-transduced 32D cell clones: it ranged between 9.6×10^6 to 1.5×10^7 molecules/μg total RNA.

Moreover, we investigated the consequences of the observed time-course increase of *bcr-abl* fusion gene transcription rates on other biomolecular pathways potentially relevant for clonal evolution of p210 *bcr-abl*-transduced 32D cells denominated B. These cell clones did not show any significant difference in growth rate in liquid culture or plating efficiency in semisolid culture medium (methylcellulose) or in cell cycle distribution. In addition, all of them exhibited Stat5 constitutive phosphorylation since the development of growth factor independence (data not shown). Interestingly, we found that the abrogation of apoptotic cell death induced by low dose/low dose rate γ irradiation occurred concomitantly with the increase of p210$^{bcr-abl}$ transcription rate, 90 days following electroporation (Figure 4).

Discussion

Gene amplification is very often associated with progression of neoplastic diseases and development of drug resistance. Accordingly, overexpression of the p210$^{bcr-abl}$ chimera, the molecular marker of CML, has been associated with more aggressive stages of the disease and resistance to interferon-α or the tyrosine kinase inhibitor, STI571.[10,11] As a consequence, the assessment of gene expression levels would represent an important tool in prognostic classification and follow-up of CML patients. To address the matter of gene amplification in clonal evolution of CML hematopoietic progenitor cells, how frequently it may take place, whether it occurs at DNA or RNA level and potential consequences on other biomolecular pathways relevant to disease progression, we sequentially measured over a period of 200 days the *bcr-abl* gene copy numbers and transcript molecules in seventeen 32D cell clones stably transducing a p210$^{bcr-abl}$ construct. The 32D hematopoietic progenitor cell line is a diploid, non-tumorigenic, IL-3-dependent cell line, which has been extensively used to assess the effects of

many oncogenes on the process of neoplastic transformation.[20] To our purpose, we developed a competitive PCR strategy, whose accuracy and reproducibility were preliminarily assayed. In fact, the technique has a sufficient sensitivity to detect 2.8×10^4 molecules of the fusion gene/100 ng of genomic DNA, and 2.25×10^6 molecules of the chimeric transcript/μg of total RNA in the $p210^{bcr\text{-}abl}$-transduced 32D LG7 cell clone, bearing a single copy of the fusion gene.[16] In all $p210^{bcr\text{-}abl}$-transduced 32D cell clones the fusion gene copy number exceeded that of 32D LG7 by 4-6 fold from cloning and selection in G418-additioned medium and remained steady over a 200-day period after electroporation (Figure 3B). This high level of gene integration probably results from the transfection technique: electroporation, in fact, probably forces more transgene copies into the cell genome in consequence of the electrical shock than the retroviral infection used to generate the 32D LG7 cell clone. The fusion gene transcription rate of all $p210^{bcr\text{-}abl}$-transduced 32D cell clones exceeded the transcript molecule number of 32G LG7 by 7.6-20.8 fold 60 days after electroporation and rose significantly further thereafter up to 28.4-42.5 fold that of the control value (Figure 3A). Our result is thus consistent with transcriptional regulation of $p210^{bcr\text{-}abl}$ as a prominent feature associated with clonal evolution of hematopoietic progenitor cells expressing the fusion gene. Nonetheless, our results do not rule out a role for gene amplification, which has been demonstrated in other experimental models.[21,25] In our study the abrogation of radiation-induced apoptotic cell death was the only other pathway associated with overexpression of $p210^{bcr\text{-}abl}$ (Figure 4). We can only speculate that the interactions between the fusion gene transcript of product and other viability-associated genes, such as Bcl-2 or Bcl-x, require a gene dosage threshold.[26] The competitive PCR strategy described in our study can be used on RT products but not on genomic DNA from hematopoietic progenitor cells from CML patients. Since the breakpoint on chromosome 9 (*c-abl* oncogene) in patients with the Ph chromosome may occur over a 100 kb region, PCR detection based on DNA as a whole may not be successful because of this large molecular distance.[27] Accordingly, other technical approaches, such as Southern blotting or FISH, must be used to assess gene amplification for clinical purposes. It is worth noting that both gene amplification and gene overexpression would result in increased levels and kinase activity of $p210^{bcr\text{–}abl}$ protein, which, in turn, might hasten disease progression toward blast crisis and induce resistance to the tyrosine kinase inhibitor, STI571. Their assessment might, therefore, serve to improve prognostic classification of CML patients at clinical diagnosis and during the follow-up.

Contributions and Acknowledgments

FC and MAS were the principal investigators involved in both the design of the study and in the writing of the paper. LP and GB performed the molecular analysis. MP provided the immunohystochemical stainings. EB and LB were responsible for the calibration of radioactive sources. ST critically revised the manuscript and gave his final approval for submission. The order of authorship was made according to single contributions given to the study.

Funding

The study was supported by the University of Bologna (fondi per la Ricerca Istituzionale, Progetti Pluriennali e ex-60%), by the Fondazione CARISBO and Forlì-Cesena AIL. FC is the recipient of a grant in memory of Mrs. Lalla Seràgnoli. Gianluca Brusa and Laura Pattacini are recipients of grants provided by the Centro Interdipartimentale per la Ricerca sul Cancro "Giorgio Prodi".

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript Processing

This manuscript was peer-reviewed by two external referees and by Prof. Francesco Lo Coco, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Prof. Lo Coco and the Editors. Manuscript received September 14, 2000; accepted December 28, 2000.

Potential implications for clinical practice

The study investigated the role of gene amplification in the progression of CML. It showed that the bcr-abl fusion gene expression levels represent a molecular pathway critical for clonal evolution in vitro, and might be relevant for prognostic classification of patients.

References

1. Fialkow PJ, Jacobson RJ, Papayannopoulou T. Chronic myelocytic leukemia. Clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet and monocyte/macrophage. Am J Med 1977; 63:125-30.
2. Kantarjian HM, Deisseroth A, Kurzrock R, Estrov Z, Talpaz M. Chronic myelogenous leukemia: a concise update. Blood 1993; 82:691-703.
3. Nowell PC, Hungerford DA. A minute chromosome in human chronic granulocytic leukemia. Science 1960; 132:1497.
4. Groffen J, Stephenson JR, Heisterkamp N, de Klein A, Bartram CR, Grosveld G. Phildelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. Cell 1984; 36:93-9.
5. Heisterkamp N, Stam K, Groffen J, de Klein A, Grosveld G. Structural organization of the bcr gene and its role in the Ph' translocation. Nature 1985; 315:758-61.
6. Shtivelman E, Lifshitz B, Gale RP, Canaani E. Fused transcript of abl and bcr genes in chronic myelogenous leukemia. Nature 1985; 313:550-4.
7. Santucci MA, Saglio G, Tura S. Pathogenesis and progression of chronic myeloid leukemia. Haematologica 1996; 81:63-76.
8. Daley GQ, Van Etten RA, Baltimore D. Induction of chronic myelogenous leukemia in mice by the p210 bcr/abl gene of the Philadelphia chromosome. Science 1990;

247:824-30.
9. Daley GQ, Van Etten RA, Baltimore D. Blast crisis in a murine model of chronic myelogenous leukemia. Proc Natl Acad Sci USA 1991; 88:11335-8.
10. Gaiger A, Henn T, Horth E, et al. Increase of BCR-ABL chimeric mRNA expression in tumor cells of patients with chronic myeloid leukemia precedes disease progression. Blood 1995; 86:2371-8.
11. le Coutre P, Tassi E, Varella-Garcia M, et al. Induction of resistance to the Abelson inhibitor STI571 in human leukemic cells through gene amplification. Blood 2000; 95:1758-66.
12. Dewald GW, Wyatt WA, Juneau AL, et al. Highly sensitive fluorescence in situ hybridization method to detect double BCR/ABL fusion and monitor response to therapy in chronic myeloid leukemia. Blood 1998; 91:3357-65.
13. Hochhaus A, Reiter A, Skladny H, Reichert A, Saussele S, Hehlmann R. Molecular monitoring of residual disease in chronic myelogenous leukemia patients after therapy. Recent Results Cancer Res 1998; 144:36-45.
14. Greenberger JS, Sakakeeny MA, Humphies KC, Eaves CG, Ekner RJ. Demonstration of a permanent factor-dependent multipotential (erythroid/neutrophil/basophil) hematopoietic progenitor cell line. Proc Natl Acad Sci USA 1983; 80:2931-5.
15. Carlesso N, Griffin JD, Druker BJ. Use of a temperature-sensitive mutant to define the biological effects of the p210 bcr-abl tyrosine kinase on proliferation of a factor-dependent murine myeloid cell line. Oncogene 1994; 9: 149-56.
16. Laneuville P, Sun G, Timm M, Vekemans M. Clonal evolution in a myeloid cell line transformed to interleukin-3 independent growth by retroviral transduction and expression of p210 bcr/abl. Blood 1992; 80:1788-97.
17. Diviacco S, Norio P, Zentilin L, et al. A novel procedure for quantitative polymerase chain reaction by coamplification of competitive templates. Gene 1992; 122:313-20.
18. Sambrook J, Fritsch EF, Maniatis T. In: C. Nolan, ed. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, New York; 1989.
19. Russell H. In: NA Erlich, ed. PCR technology: principles and applications for DNA amplification. Stockton Press, New York; 1987.
20. Rovera G, Kreider B, Shirsat N, Venturelli D, Naso G, Mavilio F. Alteration of the program of terminal differentation caused by oncogenes in the hemopoietic progenitor cell line 32D Cl3 (G). Ann NY Acad Sci 1989; 567:154-64.
21. Mahon FX, Deininger MWN, Schultheis B, et al. Selection and characterization of BCR-ABL positive cell lines with differential sensitivity to the tyrosine kinase inhibitor STI571: diverse mechanisms of resistance. Blood 2000; 96:1070-9.
22. Siebert PD, Larrick JW. Competitive PCR. Nature 1992; 359:557-8.
23. Grassi G, Zentilin L, Tafuro S, et al. A rapid procedure for the quantitation of low abundance RNAs by competitive reverse transcription-polymerase chain reaction. Nucleic Acids Res 1994; 22:4547-9.
24. Sabath DE, Broome HE, Prystowsky MB. Glyceraldehyde-3-phosphate dehydrogenase mRNA is a major interleukin 2-induced transcript in a cloned T-helper lymphocyte. Gene 1990; 91:185-91.
25. Weisberg E, Griffin JD. Mechanism of resistance to the ABL tyrosine kinase inhibitor STI571 in BCR/ABL-transformed hematopoietic cell lines. Blood 2000; 95:3498-505.
26. Cirinna M, Trotta R, Salomoni P, et al. Bcl2 expression restores the leukemogenic potential of a BCR/ABL mutant defective in transformation. Blood 2000; 96:3915-21.
27. Han JA, Lee KO, Kim YW, Kim YJ, Park YS. Detection of the BCR/abl gene rearrangement by reverse transcriptase based polymerase chain reaction. J Biochem Mol Biol 1996; 29:341-7.
28. Nishii K, Kabarowski JHS, Gibbons DL, et al. BCR-ABL kinase activation confers increased resistance to genotoxic damage via cell cycle block. Oncogene 1996; 13: 2225-34.