

## MYC in Chronic Myeloid Leukemia: Induction of Aberrant DNA Synthesis and Association with Poor Response to Imatinib

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### Abstract

Untreated chronic myeloid leukemia (CML) progresses from chronic phase to blastic crisis (BC). Increased genomic instability, deregulated proliferation, and loss of differentiation appear associated to BC, but the molecular alterations underlying the progression of CML are poorly characterized. *MYC* oncogene is frequently deregulated in human cancer, often associated with tumor progression. Genomic instability and induction of aberrant DNA replication are described as effects of *MYC*. In this report, we studied *MYC* activities in CML cell lines with conditional *MYC* expression with and without exposure to imatinib, the front-line drug in CML therapy. In cells with conditional *MYC* expression, *MYC* did not rescue the proliferation arrest mediated by imatinib but provoked aberrant DNA synthesis and accumulation of cells with 4C content. We studied *MYC* mRNA expression in 66 CML patients at different phases of the disease, and we found that *MYC* expression was higher in CML patients at diagnosis than control bone marrows or in patients responding to imatinib. Further, high *MYC* levels at diagnosis correlated with a poor response to imatinib. *MYC* expression did not directly correlate with BCR-ABL levels in patients treated with imatinib. Overall our study suggests that, as in other tumor models, *MYC*-induced aberrant DNA synthesis in CML cells is consistent with *MYC* overexpression in untreated CML patients and nonresponding patients and supports a role for *MYC* in CML progression, possibly through promotion of genomic instability. *Mol Cancer Res*; 9(5); 564–76. ©2011 AACR.

### Introduction

c-Myc (*MYC* herein after) is an oncogenic transcription factor of the helix-loop-helix/leucine zipper protein family. *MYC* is a widespread regulator of transcription that directly

or indirectly regulates about 1,000 genes, and binds to 15% of genomic loci (reviewed in refs. 1, 2). *MYC* is found deregulated in nearly half of human tumors and appears frequently associated with tumor progression (3, 4). However, as most human tumors are relatively advanced at the time of discovery it is difficult to ascertain whether *MYC* became deregulated at an early or late stage of disease progression. A number of tumor-related activities have been described for *MYC* such as the increased proliferative potential, enhanced protein synthesis, and energetic metabolism, differentiation arrest and genomic instability (2, 5). Genomic instability is thought to be essential for *MYC*-induced carcinogenesis, as shown in cell culture and mouse models (reviewed in refs. 6, 7). Mechanisms for *MYC*-mediated genomic instability include the disruption of cell-cycle checkpoints (2, 8), disruption of DNA repair (9) and unscheduled DNA replication (10–14). An increasing role of *MYC* in inducing DNA synthesis in conditions of cell stress has been gathering over the last years (reviewed in ref. 15). In cell culture models this abnormal induction of DNA synthesis often results in aberrant or "illegitimate" DNA synthesis uncoupled from cell division, leading to G2 arrest and/or polyploidy (16–18).

Chronic myeloid leukemia (CML) is a myeloproliferative disorder that represents 15% to 20% of newly diagnosed leukemias. CML progresses in 3 phases: most of the patients are diagnosed in a relatively benign chronic phase (CP) followed by an accelerated phase and finally a blastic crisis

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(BC) phase (19). The molecular hallmark of all CML phases is the expression of the BCR-ABL kinase and the BCR-ABL inhibitor Imatinib ("Gleevec") is today the frontline drug in CML therapy (20–22). However, despite the efficacy of imatinib in prolonging the CP, there is a significant fraction of patients that fail to respond and thus frequent monitoring is needed (23, 24). It is believed that CML is a stem cell malignancy in which BCR-ABL would lead to a progressive block of differentiation and increased genetic instability (22, 25–27). However, the mechanisms underlying CML progression are still uncertain. As BCR-ABL is already present in CP, it is assumed that progression is a multistep, time-dependent process that requires the mutation or deregulation of additional genes. Actually, BCR-ABL expression levels or phosphorylation of BCR-ABL substrates do not fully determine the prognosis for individual patients (25, 28). This has driven the search for other genes that could serve as molecular markers for CML progression. Different large-scale genomic profiling studies have identified a series of candidate genes, but these vary significantly across different studies (reviewed in refs. 22, 25). The involvement of MYC in CML has not been fully addressed. However, CML constitutes an interesting tumor to study MYC involvement because (i) CML begins in the more benign CP which can be significantly extended by imatinib treatment; (ii) samples from the same patient can be analyzed for MYC expression at different stages along the evolution of the leukemia; (iii) BCR-ABL upregulates MYC expression (29–31) and MYC cooperates with BCR-ABL in transformation (32–34), and (iv) MYC activities in genomic instability and differentiation arrest have been associated to CML progression (22, 25). However, MYC expression in the different CML phases and in relation to treatment response is so far unreported. Studies conducted with a small number of cases reported that MYC mRNA levels are either elevated or unchanged in CML-BC (35–39). It is also of note that trisomy 8 and gain at 8q24 (where MYC maps) are among the most frequent cytogenetic alterations in CML (40, 41).

Here we first show that, in the presence of imatinib, MYC promotes aberrant DNA replication (uncoupled from mitosis), a MYC activity related to genomic instability. Consistently, we also found increased MYC expression in untreated CML cases. Moreover, we found a positive correlation between MYC expression at diagnosis and poor response to imatinib, which is not directly dependent on BCR-ABL expression.

## Materials and Methods

### Cell lines, cell proliferation, and DNA synthesis assays

The K562 cell line, derived from CML-BC, was obtained from the American Type Culture Collection. KmycB cells are K562 cells stably transfected with inducible MYC gene (42). To generate the KMER4 cell line, K562 cells were electroporated (BioRad Gene Pulser apparatus) with pBABEPuro-mycER plasmid. This vector expresses the Myc-ER chimera, which is activated by 4-hydroxytamoxifen (4HT; ref. 43). Transfected clones were selected with 1 µg/mL of

puromycin. KLBcl2v cells are K562 cells expressing Bcl2 (44). To generate the KmycBcl2 cell line, KmycB cells were retrovirally transduced with a Bcl2 expression vector as described (44). To generate the KmycBT315I cell line, KmycB cells were electroporated with an expression vector for BCR-ABL-T315I mutant (pSRαp210T315I; ref. 45). All cell lines were grown in RPMI 1640 medium (Gibco-Life Sciences) containing 10% fetal calf serum (FCS), gentamycin (80 µg/mL), and ciprofloxacin (2.5 µg/mL). For proliferation assays, exponentially growing cells were plated at a concentration of 250,000 cells/mL on day 0. For thymidine incorporation assays, cells were incubated with 1 µCi/mL of <sup>3</sup>H-thymidine for 2 hours, harvested onto glass wool filters and the radioactivity was counted by liquid scintillation. To analyze the fraction of cells undergoing DNA synthesis, cells were cultured in the presence of 30 µmol/L bromodeoxyuridine (BrdU) for 90 minutes and processed as described (46).

### Cell cycle and apoptosis analysis

Cells were resuspended in PBS-sodium citrate buffer containing 10 µg of bovine serum albumin/mL, 200 µg of RNase/mL, and 50 µg/mL of propidium iodide (PI). The cells were incubated at 37°C in the dark for 30 minutes and then analyzed by flow cytometry using CellQuest software. For double labeling with BrdU and PI, the cells were pulsed with 10 µmol/L BrdU for 45 minutes, harvested, fixed in 75% ethanol at 4°C, washed with PBS and incubated for 20 minutes with 2N HCl, neutralized with 0.1 mol/L borate buffer, pH 8.3, washed and resuspended in PBS, 5% FCS, 0.5% Tween-20 (PFST). Cells were incubated with an anti-BrdU monoclonal antibody (BD Biosciences, diluted 1:100) for 60 minutes at RT washed, and incubated for 60 minutes with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:100, Jackson Immuno). Cells were washed, and incubated for 2 hours with 5 µg/mL PI and 50 µg/mL RNase and the cells were analyzed by flow cytometry. Apoptosis was assessed by Annexin V binding and internucleosomal DNA fragmentation assays. Annexin V binding was detected by flow cytometry using the BD-Pharmingen kit. The presence of internucleosomal DNA fragmentation (DNA laddering) after cell exposure to imatinib was analyzed by electrophoresis on a 1.5% agarose gel as previously described (47).

### Immunoblots

K562 cells and bone marrow cells were lysed with 1% NP40 and 0.2% SDS and sonicated. The protein levels were determined by immunoblot as described (46). Anti-MYC antibody (N-262, rabbit polyclonal), anti-actin (I-19, goat polyclonal) and anti-Bcl2 (C-21, rabbit polyclonal), anti ERK2 (C-14, rabbit polyclonal), and anti-α-tubulin (H-300, rabbit polyclonal) were from Santa Cruz Biotechnology.

### RNA analysis

Total RNA was prepared with TriReagent (Invitrogen). For Northern analysis, RNAs (15 µg of total RNA per lane)

were separated by electrophoresis through agarose-formaldehyde gel and transferred to nitrocellulose. Probe labeling with [ $\alpha$ - $^{32}$ P]dCTP and filter hybridization were carried out according to standard procedures. Probes for human MYC and histone H4 were as described (42). RNA levels in clinical samples were determined by quantitative reverse transcription-PCR (qRT-PCR). Reverse transcription and quantitative PCR were carried out as described (48). MYC expression was normalized against the mRNA levels of ribosomal protein S14 (RPS14). The primers for MYC were 5'-AAGACTCCAGCGCCTTCTCTC-3' and 5'-GTTTTCCAACCTCCGGGATCTG-3'. The primers for RPS14 were 5'-TCACCG-CCCTACACATCAAAC-3' and 5'-CTGCGAGTGC-TGTCAGAGG-3'. RPS14 has not been described as a MYC target gene (www.myccancer.org). Primers for BCR-ABL were as described (49). The experimental variability was controlled using a control cDNA pool synthesized with RNAs from K562 and HeLa cells (50% each).

#### Luciferase reporter assays

Three million KMER4 cells were electroporated at 260 V and 1 mFa in a Bio-Rad electroporator with 3  $\mu$ g of pGL2-M4-Luc reporter, which carries 4 E-boxes in the promoter (50) and 1  $\mu$ g of the *Renilla* luciferase vector pRL-TK (Promega). After 24 hours of incubation, cultures were split into aliquots and further incubated for 24 hours with 200 nmol/L 4HT. Cells were lysed and the luciferase activity was measured in duplicate by a dual-luciferase reporter gene assay system (Promega). Data were normalized against the *Renilla* luciferase activity.

#### CML patient samples

Bone marrow mononuclear cells from 66 CML patients (median age of 55 at the time of diagnosis) were studied. Patients were treated with imatinib and samples were taken up to 36 months of treatment. The patients are from 2 hospitals: Hospital Universitario Marqués de Valdecilla (Santander, Spain) and Hospital Universitario Dr. Negrín (Las Palmas, Spain). The origin and characteristics of each patient included in our study are summarized in Supplementary Table S1. Patients were evaluated for hematologic response (HR), complete cytogenetic response (CCR), major molecular response (MMR), and complete molecular response (CMR) and classified into optimal and suboptimal responses as described (51). Treatment failure was also recorded in 9 patients. For our statistical analyses, we have considered as nonresponder those patients that did not achieve CCR at any time across the study. This study was approved by the ethics committees according to procedures approved by the 2 hospitals providing the samples.

#### Statistical analysis

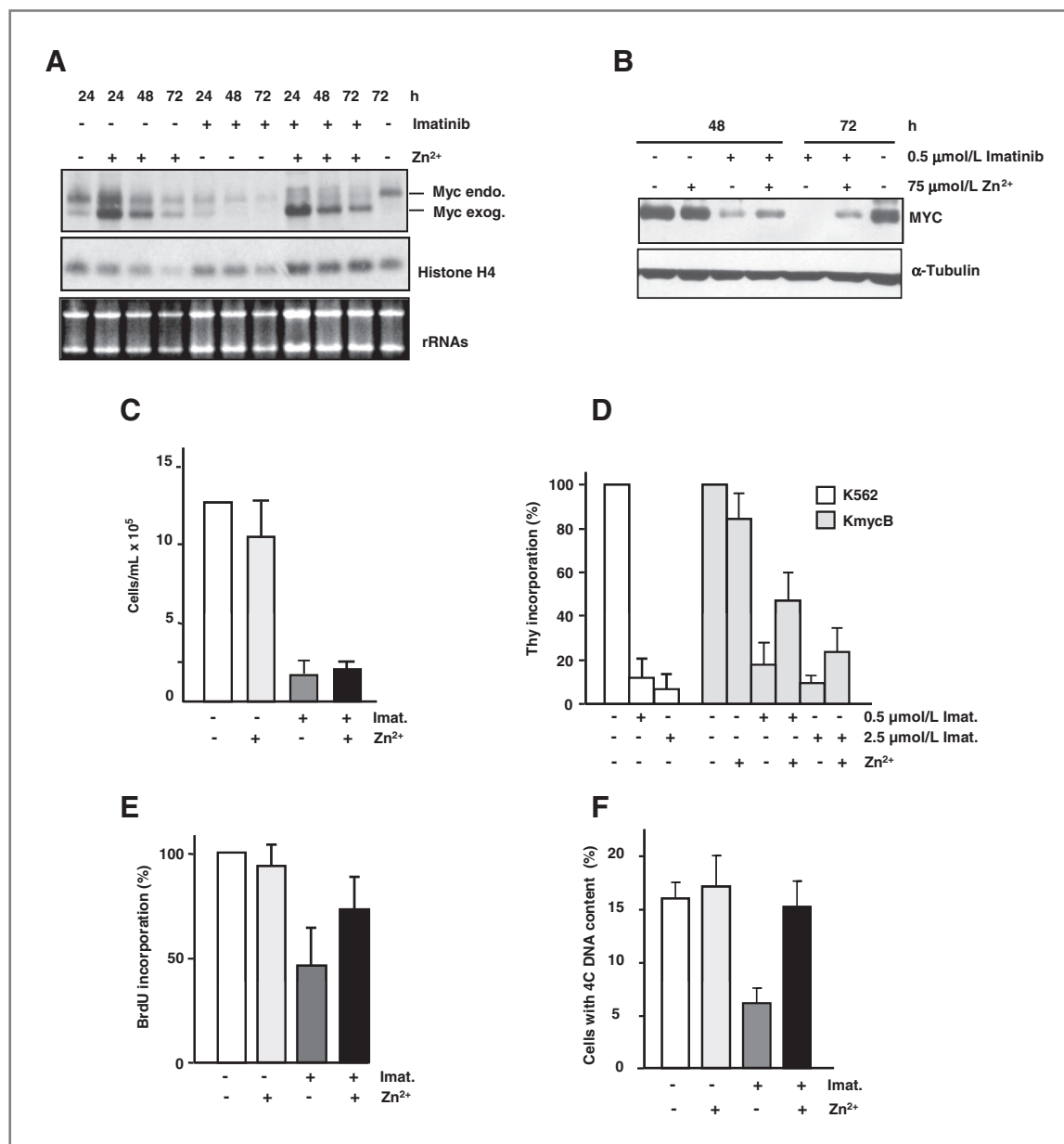
Groups were compared using the Mann–Whitney test. Cumulative rates of different types of responses were estimated according to the Kaplan–Meier method and the significance in each group was tested by log rank test.

MYC levels were dichotomized using their median value as cutoff. Patients with lack of response at the end of the follow-up were considered as censored. Relationships between MYC and CCR and MMR were analyzed using the random-effects logistic regression model, where the probabilities of CCR or MMR were estimated as functions of MYC levels using the formula  $P(\text{response}) = \frac{e^{\text{OR}}}{1 + e^{\text{OR}}}$  (52). The efficacy of MYC expression to classify patients into responders and nonresponders was studied by generating ROC curves. MYC and BCR-ABL expression were compared by Sperman's  $\rho$  and Pearson's correlation coefficient. All  $P$ -values were calculated from 2-side tests and values below 0.05 were considered significant.

## Results

### Ectopic MYC increased DNA synthesis in K562 cells treated with imatinib

We previously reported that imatinib provoked a dramatic downregulation of MYC in K562 and other CML-derived cell lines (30) and it is reported that MYC can induce unscheduled DNA synthesis in conditions of cell stress (15). Therefore, we asked whether MYC could reverse the arrest in DNA synthesis and cell growth provoked by imatinib. We used the KmycB cell line, a derivative of the CML cell line K562 carrying a Zn $^{2+}$ -inducible MYC allele (42). As reported for parental cells, imatinib repressed MYC (30), but the addition of the inducer (ZnSO $_4$ ) increased exogenous MYC mRNA levels even in the presence of imatinib in KmycB cells (Fig. 1A). The decrease in MYC 24 to 48 hours after ZnSO $_4$  addition is already reported (42). It is due to the inducible system used, based on the metallothionein promoter, and occurs with other genes driven by this promoter in K562 cells (47, 53). MYC protein was also detected at significant levels in KmycB cells treated with ZnSO $_4$  and imatinib, assessed by immunoblot (Fig. 1B). It is important to note that, in this model system, the levels of MYC mRNA and protein achieved on induction in the presence of imatinib are not supraphysiologic but similar to or lower than those of control untreated cells. However, MYC did not modify the proliferation arrest mediated by imatinib (Fig. 1C). We next asked whether MYC could induce DNA synthesis uncoupled from mitosis, a marker of MYC activity in genomic instability (see the Introduction section). DNA synthesis, determined by  $^3$ H-thymidine incorporation, was halted in parental cells after imatinib treatment but significantly increased on induction of MYC in KmycB cells in the presence of imatinib (Fig. 1D). The increased DNA synthesis in imatinib-treated cells on MYC induction was also observed by measuring DNA synthesis through the incorporation of BrdU in KmycB cells (Fig. 1E). Similar results were obtained with KmycJ cells, another MYC-inducible K562 line (ref. 42; data not shown). As mRNA synthesis of the nucleosomal histones genes is concomitant with DNA replication, we determined mRNA levels of histone H4. We found that the stimulation of DNA synthesis mediated by MYC was accompanied by



**Figure 1.** MYC induces aberrant DNA synthesis in the presence of imatinib in KmycB cells. A, induction of MYC mRNA expression in KmycB cells in the presence of imatinib. Cells were treated with 0.5  $\mu\text{mol/L}$  imatinib and 75  $\mu\text{mol/L}$   $\text{ZnSO}_4$  for 24, 48, or 72 hours as indicated. MYC and histone H4 mRNA levels were determined by northern analysis. A picture of the filter after transfer showing the rRNAs stained with ethidium bromide is shown in each case to assess the loading and integrity of the RNAs. B, induction of MYC expression in KmycB cells in the presence of imatinib. Cells were treated as in (A) for 48 or 72 hours. Protein extracts were analyzed by immunoblotting with antibodies to MYC and  $\alpha$ -tubulin as a loading control. C, cell proliferation of KmycB cells treated with 0.5  $\mu\text{mol/L}$  imatinib and 75  $\mu\text{mol/L}$   $\text{ZnSO}_4$  as in A. D, DNA synthesis measured by thymidine (Thy) incorporation. Cells were treated for 72 hours with imatinib and DNA synthesis was determined by  $^3\text{H}$ -thymidine incorporation. Data are mean values from 4 experiments, and relative to the incorporation in untreated cells at each time point; bars indicate SEM. E, DNA synthesis measured by BrdU incorporation. KmycB cells were treated with 0.5  $\mu\text{mol/L}$  imatinib and 75  $\mu\text{mol/L}$   $\text{ZnSO}_4$  for 72 hours. Data are mean values from 4 experiments. F, MYC induces an accumulation of cells with 4C DNA content in the presence of imatinib. KmycB cells were treated for 48 hours with 0.5  $\mu\text{mol/L}$  imatinib and 75  $\mu\text{mol/L}$   $\text{ZnSO}_4$  and the fraction of live cells in each cell-cycle phase was determined by PI staining. The fraction of cells with 4C DNA content is indicated in each case. The data are mean values from 5 independent experiments. Bars indicate SEM.

the upregulation of histone H4 mRNA as shown by northern analysis (Fig. 1A).

As MYC induced DNA synthesis but not mitosis in the presence of imatinib in KmycB cells, we expected that MYC

induced accumulation of cells with more than 2C DNA content, as reported in other models (16–18). This was indeed observed by cell-cycle analysis in KmycB, which showed a higher fraction of cells with 4C DNA content in

the cells treated with imatinib and ZnSO<sub>4</sub> with respect to cells treated only with imatinib (Fig. 1F and Supplementary Fig. S1). This effect is clearly detected after 48 hours of imatinib treatment. However, at longer treatment intervals with 0.5 μmol/L imatinib a significant fraction of cells undergo apoptosis (data not shown).

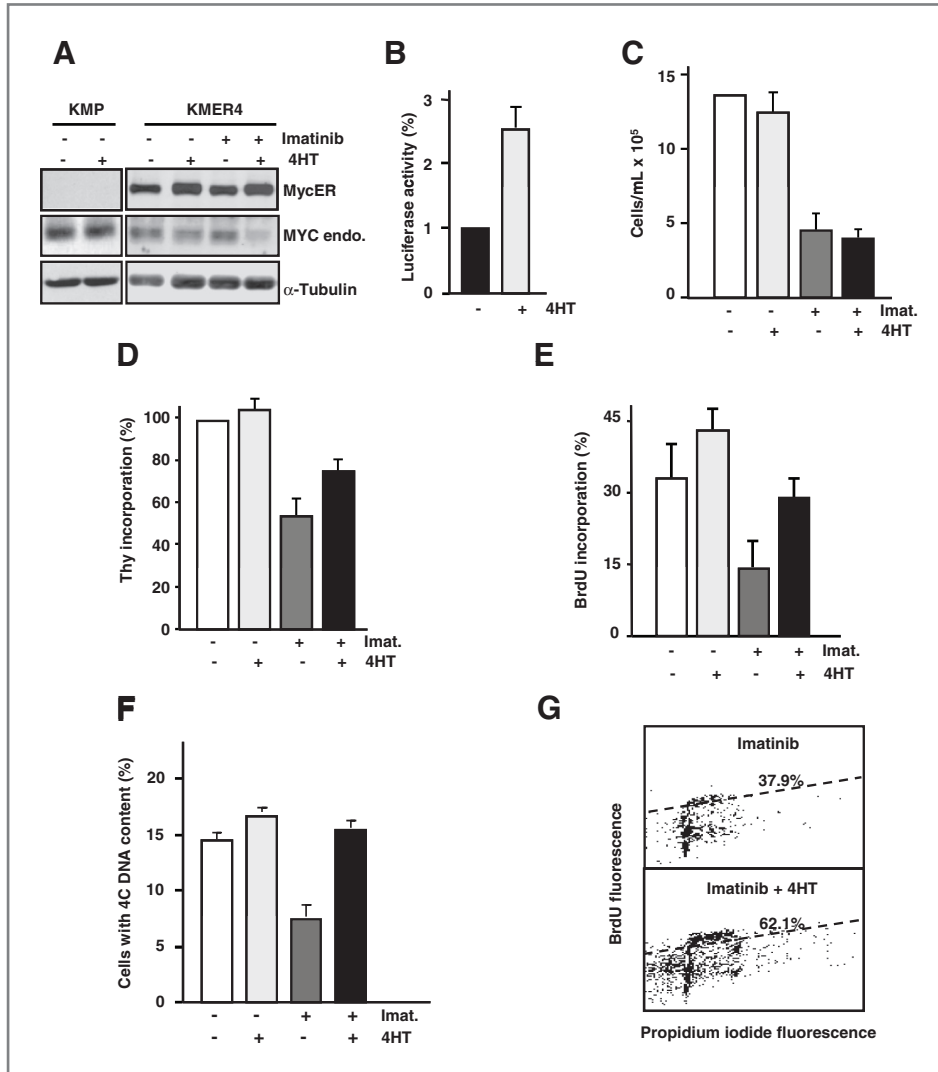
The previous results showed that MYC induced DNA synthesis in the presence of imatinib in cell lines with Zn-inducible MYC. To more rigorously assess the role of MYC in this process we wanted to test a different system of conditional MYC expression. For this purpose, we generated a K562 derivative, termed KMER4, expressing a chimerical protein with MYC fused with the hormone binding region of the estrogen receptor (MycER; ref. 43). Immunoblot analysis showed the expression of MycER at high levels (Fig. 2A). The activation of MycER by 4-hydroxytamoxifen (4HT) in KMER4 cells was assessed, first, by the MYC downregulation of endogenous MYC (Fig. 2A), an effect observed in many cell lines, including K562 (42, 54). We further confirmed the activation of MycER by 4HT through transactivation assays of a luciferase reporter carrying 4 MYC-responsive E-boxes (Fig. 2B). Similarly to the previous observation on KmycB cells, the activation of MYC by 4HT did not rescue the proliferation arrest elicited by imatinib in KMER4 cells (Fig. 2C). However, MYC activation augmented the DNA synthesis as measured by <sup>3</sup>H-thymidine incorporation (Fig. 2D) as well as by BrdU incorporation (Fig. 2E). The analysis of DNA content by PI staining and flow cytometry showed an accumulation of cells with 4C DNA content (Fig. 2F and Supplementary Fig. S1). We carried out a double staining of PI and BrdU in KMER cells treated with imatinib and 4HT. The results confirmed that the cells with 4C content also incorporated BrdU (Fig. 2G). Thus, the results in KMER4 cells are similar to those observed in KmycB, i.e., that conditional activation of MYC provoked aberrant DNA synthesis in cells exposed to imatinib. Like many CML-derived cell lines, K562 carries mutated *TP53* alleles. Nonetheless, an important fraction of CML in BC carry wild-type *TP53* (25) and a dependence on p53 for MYC-driven genomic instability has been reported in some models (55–57). However, using a K562 derivative with conditional p53 expression (50), we found that MYC can also induce G2 accumulation in the presence of imatinib in cells with active p53 (Supplementary Fig. S2). BCR-ABL induces MYC levels through JAK2 and JAK2 regulates BCR-ABL signaling (29, 31). However, MYC activation in our K562 models did not reverse the JAK2 inactivation induced by imatinib (not shown).

The former results show a MYC-mediated accumulation of cells with 4C DNA content when cells are exposed to imatinib and MYC is activated, but, noticeably, MYC did not induce cell proliferation. A possible explanation of this result is that MYC is stimulating proliferation which is balanced by MYC-mediated apoptosis. We analyzed the effect of MYC on imatinib-mediated apoptosis by determining the fraction of cells with a sub-diploid DNA content by flow cytometry of PI-stained cells. The results showed

that imatinib induced apoptosis (25%–30% of apoptotic cells after 48 hours) but MYC did not modify this result in KmycB cells (Fig. 3A). We sought to confirm this result assessing apoptosis by a different method, i.e., the binding to Annexin V. The results again showed that MYC did not significantly increase apoptosis induced by imatinib (Fig. 3B). DNA laddering assays further confirmed this result (Fig. 3C). The lack of increased apoptosis in cells treated with imatinib and with activated MYC was not surprising as the MYC levels achieved in ZnSO<sub>4</sub>-treated cells were not supraphysiologic, due to the autoregulatory effects of MYC described above (42, 54).

The results suggest that MYC induces aberrant DNA synthesis in the presence of imatinib. Next, we wanted to determine whether the MYC effect depends on imatinib-mediated inhibition of BCR-ABL kinase activity *per se* or whether it required the proliferation arrest. To address this question we followed 2 approaches. First, we generated a KmycB derivative with constitutive expression of the BCR-ABL-T315I mutant, which is resistant to imatinib (59). These cells were highly resistant to the antiproliferative effects of imatinib (IC<sub>50</sub> ~ 7 μmol/L). However, endogenous MYC was not downregulated by imatinib and the induction of MYC did not modify the cell-cycle profile of these cells treated with imatinib, despite that endogenous wild-type BCR-ABL was inhibited (Supplementary Fig. S3). Similarly, in K562R cells, which are resistant to imatinib due to LYN kinase overexpression (60), MYC was not downregulated by imatinib despite BCR-ABL inhibition by imatinib (not shown). In the second approach, we generated a KmycB derived cell line overexpressing Bcl2, termed KmycBcl2. In contrast to K562, which does not express Bcl2 (44), KmycBcl2 expressed high levels of Bcl2 as shown by immunoblot (Fig. 3D). We also showed that, in KmycBcl2 cells, MYC was efficiently induced by ZnSO<sub>4</sub> in the presence of imatinib (Fig. 3E). Neither imatinib treatment nor MYC induction by ZnSO<sub>4</sub> affected the expression the Bcl2 transgene (Fig. 3D). KmycBcl2 cells were resistant to imatinib-mediated apoptosis (not shown), as previously reported for KLbcl2v cells (30). In concordance, KmycBcl2 cells were partly resistant to the antiproliferative effects of imatinib, as shown by cell counting (Fig. 3F) and by <sup>3</sup>H-thymidine incorporation (Fig. 3G). Nonetheless, MYC induction did not modify the effect of imatinib on proliferation (Fig. 3F). The induction of MYC in KmycBcl2 cells again resulted in augmented DNA synthesis as assessed by <sup>3</sup>H-Thy incorporation (Fig. 3G). We also determined the DNA content in KmycBcl2 cells by PI staining and we found that the fraction of cells with 4C DNA content was increased on MYC induction (Fig. 3H). Noticeably, the cell fraction with 4C content was lower in KmycBcl2 than in KmycB cells, consistent with the partial resistance of KmycBcl2 cells to the antiproliferative effect of imatinib, as compared to cells without Bcl2 overexpression.

Taken together, our results indicate that MYC induces unscheduled DNA synthesis in the presence of cell proliferation arrest induced by imatinib.

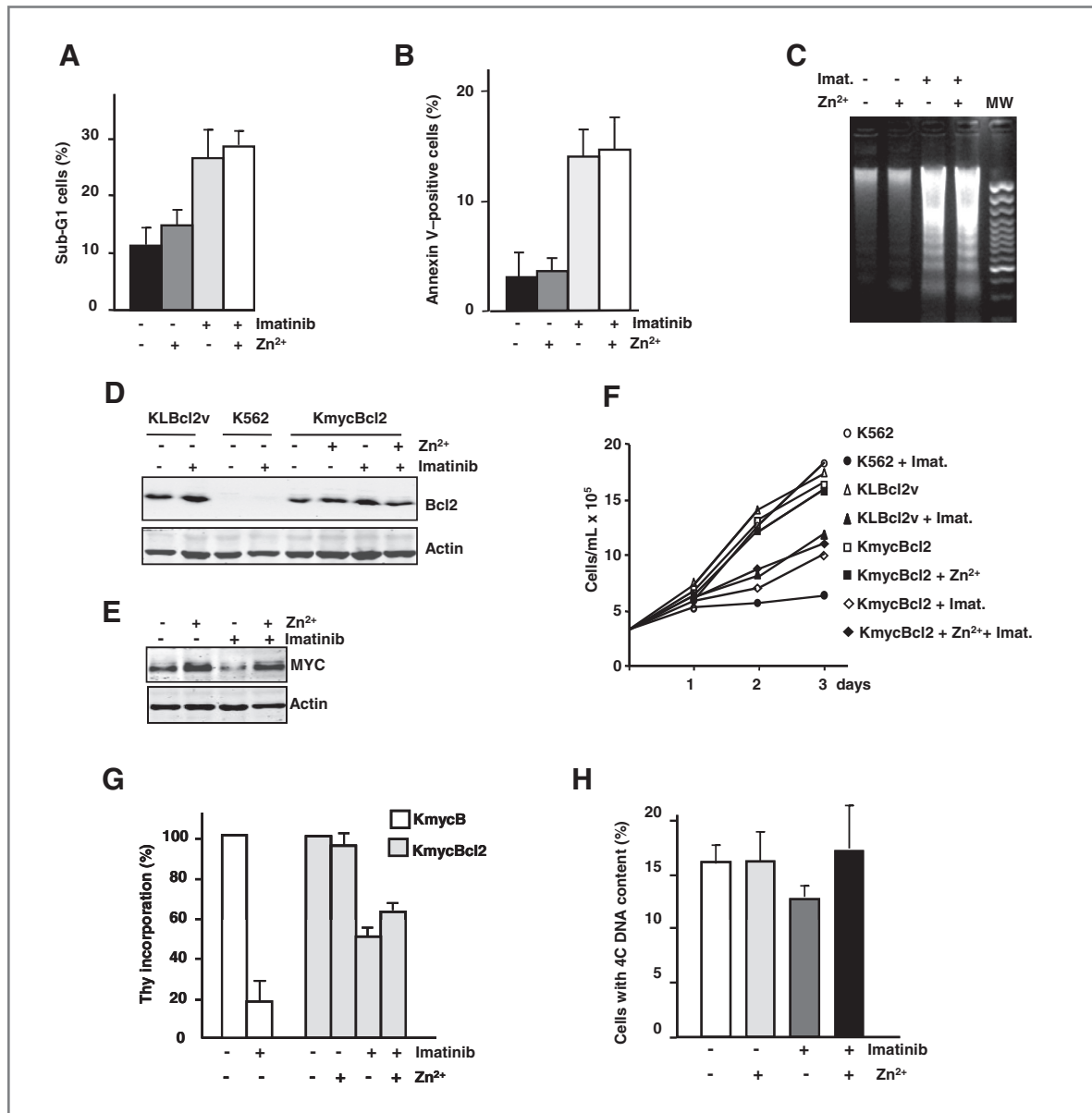


**Figure 2.** MYC induces aberrant DNA synthesis in the presence of imatinib in KMER4 cells. A, expression of MycER in KMER4 cells. Where indicated, cells were treated for 48 hours with 0.5  $\mu\text{mol/L}$  imatinib and 200 nmol/L 4HT. The levels of the fusion protein MycER and the endogenous MYC were determined by immunoblot. Levels of  $\alpha$ -tubulin were also determined to assess the protein loading. B, activation of MYC in KMER4 cells. The activation of the MycER protein by 4HT was assayed by a luciferase assay using a promoter-luciferase construct containing 4 MYC-responsive elements (pGL2-M4-luc) upstream of the firefly luciferase gene. Twenty-four hours after transfection, cells were treated with 200 nmol/L 4HT for an additional 24 hours and the luciferase activity determined, normalized to the *Renilla* luciferase internal control. Data represent the mean of 3 independent experiments; bars indicate SEM. C, cell proliferation assay of KMER4 cells treated with 0.5  $\mu\text{mol/L}$  imatinib and 200 nmol/L 4HT for 72 hours. D, DNA synthesis measured by thymidine (Thy) incorporation. Cells were treated for 72 hours with imatinib and DNA synthesis was determined by  $^3\text{H}$ -thymidine incorporation. Data are mean values from 3 experiments, and relative to the incorporation in untreated cells at each time point; bars indicate SEM. E, DNA synthesis measured by BrdU incorporation. KMER4 cells were treated with 0.5  $\mu\text{mol/L}$  imatinib and 200 nmol/L 4HT for 72 hours. Data are mean values from 3 experiments. F, MYC induces an accumulation of cells with 4C DNA content in the presence of imatinib. KMER4 cells were treated for 48 hours with 0.5  $\mu\text{mol/L}$  imatinib and 200 nmol/L 4HT and the fraction of live cells in each cell-cycle phase was determined by PI staining. The fraction of cells with 4C DNA content is indicated in each case. The data are mean values from 3 independent experiments. G, MYC induces DNA synthesis in 4C DNA cells treated with imatinib. Double labeling with BrdU and PI showing DNA synthesis in G2 cells. KMER4 cells were treated for 48 hours with 0.5  $\mu\text{mol/L}$  imatinib and 200 nmol/L 4HT (KMER4 cells). The percentage of cells with BrdU incorporated (above unlabeled controls) is indicated in each case.

### MYC expression in CML patients

The above results suggest the possibility that MYC could promote genomic instability in CML cells *in vivo* and therefore be upregulated during CML progression. To explore this hypothesis, we first compared MYC expression levels in bone marrow mononuclear cells from patients at diagnosis, CP

patients that responded to imatinib, CP patients that did not respond [i.e., not achieving complete cytogenetic response (CCR)] and BC patients (66 patients in total). The samples were provided by 2 different hospitals and their relevant clinicopathologic features are shown in Table 1. MYC mRNA levels were determined by qRT-PCR and the results showed



**Figure 3.** MYC does not increase imatinib-induced apoptosis and imatinib-mediated proliferation arrest is required for MYC-induced DNA synthesis. A, KmycB cells were treated with 0.5  $\mu\text{mol/L}$  imatinib and 75  $\mu\text{mol/L}$  ZnSO<sub>4</sub> for 48 hours and the fraction cells with sub-G1 DNA content was determined by PI staining and flow cytometry. The data are mean values from 3 independent experiments. Bars indicate SEM. B, the fraction of cells with cell surface Annexin V binding was determined by flow cytometry with Annexin V-FITC. The data are mean values from 3 independent experiments. Bars indicate SEM. C, internucleosomal DNA fragmentation assay for KmycB cells treated with 0.5  $\mu\text{mol/L}$  imatinib (Imat.) and 75  $\mu\text{mol/L}$  ZnSO<sub>4</sub> for 48 hours as indicated. D, KmycBcl2 cells were treated for the 48 hours with 0.5  $\mu\text{mol/L}$  imatinib and 70  $\mu\text{mol/L}$  ZnSO<sub>4</sub> and analyzed by immunoblot. Lysates of K562 and KLBcl2v were also included as negative and positive controls, respectively. E, immunoblot analysis showing the expression of MYC and actin (as loading control) in KmycBcl2 cells treated with 0.5  $\mu\text{mol/L}$  imatinib and 70  $\mu\text{mol/L}$  ZnSO<sub>4</sub> as indicated. F, cell proliferation curve showing the partial resistance to imatinib of KmycBcl2. The cells were treated with 70  $\mu\text{mol/L}$  ZnSO<sub>4</sub> and 0.5  $\mu\text{mol/L}$  imatinib as indicated and counted up to 72 hours. K562 and KLBcl2v were also included as controls. G, DNA synthesis measured by thymidine incorporation. Cells were treated for 72 hours with imatinib and DNA synthesis was determined by <sup>3</sup>H-thymidine incorporation. Data are mean  $\pm$  SEM from 3 experiments, and relative to the thymidine incorporation in untreated cells at each time point. The data corresponding to KmycB are the same of Figure 1, repeated here for better comparison. H, MYC induces an accumulation of cells with 4C DNA content in the presence of imatinib. KmycBcl2 cells were treated for 48 hours with 0.5  $\mu\text{mol/L}$  imatinib and 75  $\mu\text{mol/L}$  ZnSO<sub>4</sub> and the fraction of live cells in each cell-cycle phase was determined by PI staining. The fraction of cells with 4C DNA content is indicated in each case. The data are mean values from 3 independent experiments.

that MYC expression was 2- to 5-fold higher in patients at diagnosis and nonresponders than in patients responding to imatinib treatment or healthy individuals (Fig. 4A).

These results suggest that MYC could serve as a predictive marker for the clinical response to imatinib. CML natural history allows the determination of MYC levels in the same

**Table 1.** Clinicopathologic characteristics of CML patients

	<i>n</i>
Gender	
Female	28
Male	38
Mean age at diagnosis	54 y (range, 26–83)
Bcr-Abl transcript	
p210 b3a2	50
p210 b2a2	14
p210 b2a2 and p190	2
Stage at the end of the study	
CP	57
Accelerated phase	0
BC	9
Response to treatment at the end of the study by cytologic, cytogenetic, and molecular status	
Too early to evaluate response	3
HR	8
mCR	7
CCR	7
MMR	24
CMR	17
Response to treatment at the end of the study [as ELN recommendations (51)]	
Too early to evaluate response	3
Failure	9 (8 HR, 1 mCR)
Suboptimal response	10 (6 mCR, 1 MCR, 1 CCR, 1 MMR, 1 CMR)
Optimal response	40 (6 CCR, 21 MMR, 13 CMR)
No data to evaluate response with these criteria	4 (1 CCR, 2 MMR, 1 CMR)
Treatment	
Imatinib only	31
IFN first line + imatinib	22
HU/BU first line + imatinib	13

Abbreviation: mCR, minor cytogenetic response.

patient throughout the leukemia progression. To test the former hypothesis, we carried out serial determinations of *MYC* expression during disease evolution in 60 patients. We found that in most of the patients (40 of 45) that responded to imatinib (using CCR as response criteria), *MYC* expression decreased with treatment or was kept at low levels. In contrast, *MYC* levels did not decrease in non responders. Furthermore, *MYC* expression increased in most of the non responders (11 of 15) during CML progression. Some representative cases are shown in Figure 4B. The bimodal profile of *MYC* expression is noteworthy in patient 57P, who

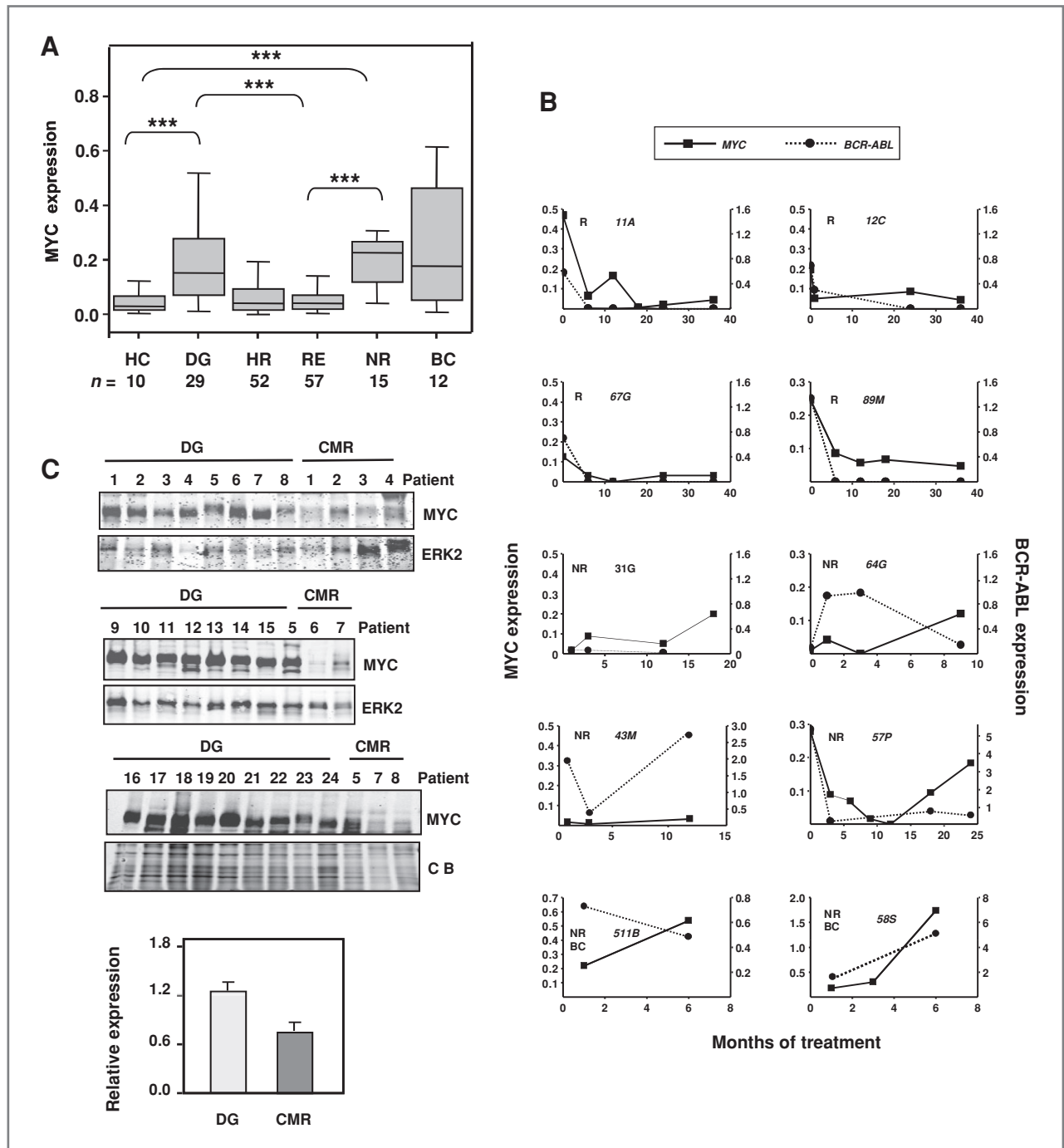
initially responded to imatinib but after 12 months became resistant to treatment as the cells expressed a BCR-ABL G250E mutation. To our knowledge this is the first report showing *MYC* overexpression during the clinical course of CML in single patients. In some settings a posttranscriptional regulation of *MYC* has been described (2, 61). Thus we analyzed the expression of *MYC* at the protein level in bone marrow samples from 32 CML patients, 24 at diagnosis and 8 at complete molecular response (CMR). Although the number of samples analyzed was small, the results show that the levels of *MYC* protein in the group of samples at diagnosis is clearly higher than in the patients at CMR (Fig. 4C). There was concordance between protein and mRNA levels in 9 of 12 samples where mRNA could be analyzed (not shown). Thus, the results indicate that the increased *MYC* expression in CML occurs also at the protein level.

### MYC mRNA levels are higher in nonresponders

Low *MYC* levels in CML could reflect the disappearance of leukemic cells in the bone marrow sample. Thus we analyzed the probability of response to imatinib as a function of *MYC* levels in CML patients. We analyzed the relationship between *MYC* levels and CCR as estimated via a random-effects logistic regression model. The results (Fig. 5A) show that relationship between *MYC* levels and CCR was odds ratio (OR) = 0.92 by each 0.01 units of *MYC*, with a 95% CI = 0.87–0.97. The relationship between *MYC* levels and major molecular response (MMR) was OR = 0.82 (95% CI: 0.73–0.94). The estimated probabilities of response to treatment as a function of *MYC* levels were  $P = 0.002$  and  $0.003$  for patients achieving CCR and MMR, respectively (Fig. 5A). Thus, we found a relationship between high *MYC* expression and lack of response (CCR and MMR; Fig. 5A), which was more robust when MMR was used as the response criterion.

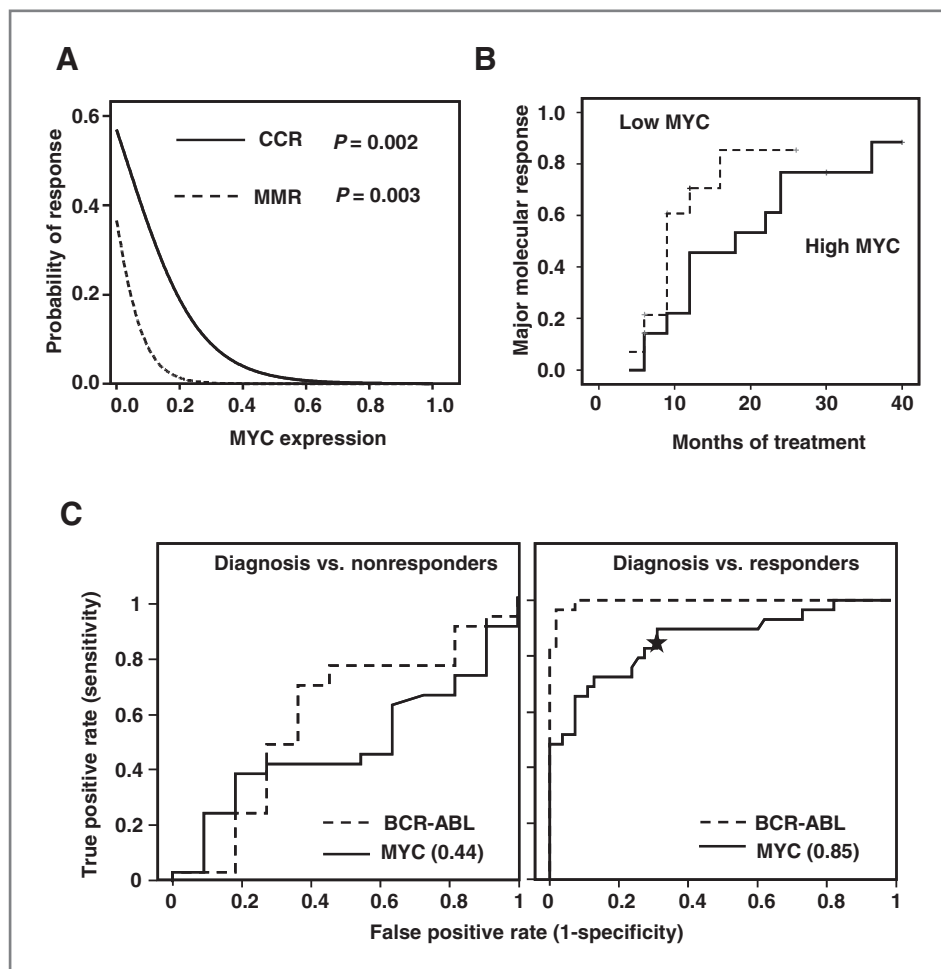
Next we asked whether *MYC* levels at diagnosis could differentiate between responders and nonresponders in a prediction model. The results showed that response (MMR) was achieved faster in patients with low *MYC* expression than in those with high *MYC* expression (Fig. 5B), although the difference did not reach significance ( $P = 0.142$ ). The former results strongly suggested an association of high *MYC* levels with poor or late response to treatment. To determine whether *MYC* expression could classify responder and nonresponder patients we compared receiver operating characteristic (ROC) curves with the data of *MYC* at diagnosis and *MYC* after treatment in responders and nonresponders. The results showed that *MYC* expression classified the patients as responders to treatment with remarkable specificity and sensitivity (area under the curve = 0.85; Fig. 5C, right), whereas it did not mark any difference between patients at diagnosis and nonresponders (Fig. 5C, left). As a control, expression of BCR-ABL was also plotted. The analysis of the ROC curves allows the selection of a *MYC* cutoff value of 0.058 with a sensitivity of 0.89 and specificity of 0.65. Remarkably, this cutoff is consistent with the mean *MYC* value of healthy controls (HC) and responders (Fig. 4A).





**Figure 4.** MYC expression in CML patients. A, MYC mRNA expression between samples from CML patients at different stages of the disease. Each box refers to the range defined by the 25th and the 75th percentiles and the line indicates the median value. DG, diagnosis; HR, HR within the first 3 months of treatment; RE, response to imatinib (at least CCR); NR, no response to imatinib; BC, number of RNA samples.  $***, P < 0.001$ . The mean of 3 replicates are plotted. B, MYC expression during CML evolution. The graphs show the expression of MYC and BCR-ABL during treatment of 10 representative patients, including 4 that responded (R) and 6 patients that did not respond to imatinib (NR). These include 2 BC. Note that the scales are different. Patient's identification appears in cursive. C, top, immunoblots showing the MYC protein levels in lysates from bone marrow cells from CML patients at diagnosis (DG) and at CMR as indicated. Bottom, quantification of the MYC protein signals with respect to ERK2 (as protein loading control) or total proteins stained with Coomassie Blue (CB). The data are mean values and bars indicate SEM.

**Figure 5.** MYC levels are higher in nonresponder patients, probability of response (CCR and MMR) after 18 months of treatment as function of MYC mRNA levels. Data are from 36 patients in CCR and 24 patients in MMR. Probabilities were estimated using random-effects logistic regression. B, response to imatinib treatment according to MYC expression at diagnosis. The MYC levels were classified as "high" or "low" with respect to the median value at diagnosis (0.153) and the MYC levels were plotted against the response to imatinib during treatment ( $n = 28$ ). C, MYC as a marker of the response to imatinib. ROC curves were plotted for MYC and BCR-ABL expression in patients at diagnosis and in responders (CCR, MMR, or CMR; right) or in patients that did not respond to treatment (left). The area under the MYC curve is indicated in each case. The star marks the MYC cutoff value (0.058 with a sensitivity of 0.89 and specificity of 0.65).



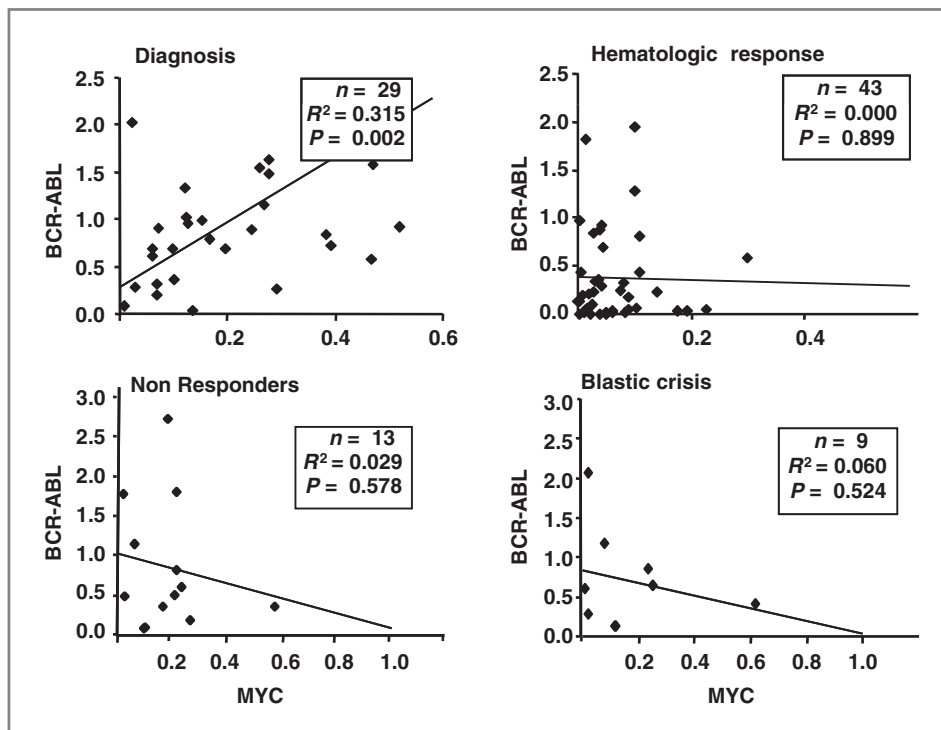
### MYC expression does not depend on BCR-ABL mRNA levels

Cell culture data indicated that BCR-ABL kinase activity induces MYC and imatinib downregulates MYC in CML-BC-derived cell lines. In clinical samples, it is expected that high BCR-ABL mRNA expression correlates with high kinase activity. So it was conceivable that MYC levels in CML patient cells correlate to those of BCR-ABL, which decrease dramatically on imatinib treatment. However, this correlation has not yet been explored in clinical samples. We compared the expression of both genes in our patients and found a positive correlation in samples at diagnosis (Fig. 6). In contrast we did not find such correlation in patients undergoing a hematologic response (HR) where a significant number of samples with high BCR-ABL showed relatively low MYC expression (Fig. 6). The lack of correlation was also observed in nonresponders and BC samples, although in these cases only 9 samples were analyzed (Fig. 6). Therefore, MYC and BCR-ABL levels did not correlate in CML patients receiving imatinib treatment. Our results rather suggest that MYC levels in CML patients inversely correlate with the normalization of hematopoiesis

and disappearance of leukemic cells, as already marked by the HR.

### Discussion

In this work, we describe 4 novel findings: (i) MYC induces unscheduled or aberrant DNA synthesis in CML cells under imatinib stress; (ii) MYC expression is higher in untreated CML patients and in those not responding to imatinib treatment; (iii) MYC levels at diagnosis could predict the response of the disease to treatment; (iv) the lack of a universal correlation between MYC and BCR-ABL expression in CML patients. In 2 CML-derived K562 cell lines with conditional MYC expression (induced by  $Zn^{2+}$  or activated by 4HT) we found that ectopic MYC expression did not antagonize imatinib-mediated growth arrest. However, in cells treated with imatinib, MYC induced both aberrant DNA synthesis uncoupled from mitosis, and a moderate accumulation of cells with 4C DNA content. Both activities have been associated to MYC-mediated genomic instability (10–13, 16–18). Taken together, the results argue for a role of MYC inducing illegitimate DNA



**Figure 6.** Lack of correlation between *MYC* and *BCR-ABL* expression in CML patients. Relationship between *BCR-ABL* and *MYC* levels in patients at diagnosis, in hematological response, nonresponders, and in BC. The solid line indicates the linear prediction. Each point represents the mean of 2 or 3 mRNA determinations for each gene. Spearman's Rho and Pearson's Correlation Coefficient are indicated in each case.

synthesis in K562 cells under imatinib-mediated stress. The promotion of DNA replication in cells subjected to stress has been identified as a major *MYC* oncogenic activity (reviewed in ref. 15).

We detected a 2- to 5-fold increase in *MYC* expression in patients at diagnosis as compared to HCs and *MYC* was also increased in patients that failed to respond to imatinib treatment. A number of previous reports indicate that this expression difference can be relevant for *MYC*-dependent carcinogenesis. For instance, just a 2-fold change means a major difference for *MYC* ability to transform cells in different cell culture models (62–64) as well as in transgenic animals where *MYC* dosage can be modulated (65). It is also noteworthy that in Burkitt lymphoma, the paradigm of *MYC* activation in human cancer, *MYC* increase in expression can be only 2-fold with respect to normal lymphocytes (66). It is surprising that *MYC* involvement in CML has gone unnoticed up to now, although the microarray expression data of a previous study show increased *MYC* expression in  $CD34^+$  cells from 9 CML patients, as compared to HCs (39). Other microarray studies have detected upregulation of *MYC* downstream genes (67–69). A report described similar expression changes in CML progression for *BMI1* (70), a polycomb group gene that cooperates with *MYC* in carcinogenesis (71), which was also undetected in microarray-based studies.

The involvement of *MYC* in CML progression suggested by our data is consistent with published data showing the relevance of *MYC* in experimental myeloid leukemia. For instance, in mice where *MYC* expression is directed to the hematopoietic precursors these animals develop acute

myeloid leukemia (72). Also, infection of murine bone marrow with *MYC* retroviruses results in myeloid but not lymphoid leukemia (73, 74). Moreover, analysis in transgenic models shows that *MYC* is essential for normal differentiation of myeloid stem cells (reviewed in ref. 75).

How could *MYC* contribute to CML progression? Our data showing *MYC* overexpression during CML progression is consistent with our studies in CML BC cells, where *MYC* promotes aberrant DNA synthesis under imatinib stress. CML progression is associated to increased genomic instability and differentiation arrest, 2 major and well-known *MYC* activities (reviewed in refs. 6, 7, 76). Thus, our data are consistent with the finding that higher *MYC* levels at diagnosis correlate with worse response to imatinib. Collectively, our results suggest a role for *MYC* in CML progression. It is conceivable that clones with higher *MYC* expression are selected during CML progression and that high-*MYC* cells are more prone to progress to BC. Further investigation is required to evaluate the usefulness of *MYC* in the assessment of CML prognosis.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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