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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. Increased genomic instability, deregulated proliferation and loss of differentiation appear associated to blastic crisis, 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 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 non-responding patients and supports a role for MYC in CML progression, possibly through promotion of genomic instability.

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 one thousand genes, and binds to 15% of genomic loci [reviewed in (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 demonstrated in cell culture and mouse models [reviewed in (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 (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 15-20% of newly diagnosed leukemias. CML progresses in three phases: most of the patients are diagnosed in a relatively benign chronic phase (CP) followed by an accelerated phase and finally a blastic crisis (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 (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 chronic phase 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 up-regulates 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 performed 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 demonstrate 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.

Results

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

We previously reported that imatinib provoked a dramatic down-regulation 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 24-48 h 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 (43, 44). 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 upon induction in the presence of imatinib are not supra-physiological but similar or lower to 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 Introduction). DNA synthesis, determined by ^{3}H-thymidine incorporation, was halted in parental cells after imatinib treatment but significantly increased upon induction of MYC in KmycB cells in the presence of imatinib (Fig. 1D). The increased DNA synthesis in imatinib-treated cells upon 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 (42) (data not shown). As mRNA synthesis of the nucleosomic 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 the up-regulation 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 >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 $ZnSO_4$ with respect to cells treated only with imatinib (Fig. 1F and Supplementary Fig.S1). This effect is clearly detected after 48 h of imatinib treatment. However, at longer treatment intervals with 0.5 μM 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) (45). Immunoblot analysis demonstrated the expression of MycER at high levels (Fig. 2A). The activation of MycER by 4HT in KMER4 cells was assessed, first, by the MYC down-regulation of endogenous MYC (Fig. 2A), an effect observed in many cell lines, including K562 (42, 46). We further confirmed the activation of MycER by 4HT through transactivation assays of a luciferase reporter carrying four 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 ^{3}H-thymidine incorporation (Fig. 2D) as well as by BrdU incorporation (Fig. 2E). The analysis of DNA content by PI staining and flow cytometry demonstrated 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 blast crisis carry wild-type TP53 (25) and a dependence on p53 for MYC-driven genomic instability has been reported in some models (47-49). 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 MCY levels through JAK2 and that JAK2 regulates BCR-ABL signalling (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 analysed 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 h) 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 $ZnSO_4$-treated cells were not supraphysiological, due to the autoregulatory effects of MYC described above (42, 46).

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 two approaches. First, we generated a KmycB derivative with constitutive expression of the BCR-ABL-T315I mutant, which is resistant to imatinib (51). These cells were highly resistant to the antiproliferative effects of imatinib (IC50 $\sim$ 7 μM). 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 (52), 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 (53), KmycBcl2 expressed high levels of Bcl2 as shown by immunoblot (Fig. 3D). We also showed that, in KmycBcl2 cells, MYC was efficiently induced by $ZnSO_4$ in the presence of imatinib (Fig. 3E). Neither imatinib treatment nor MYC induction by $ZnSO_4$ 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 ^{3}H-thymidine incorporation (Fig. 3G). Nonetheless, MYC induction did not modify the effect of imatinib on proliferation (Fig. 3F). The induction of MYC in KmycBcl2cells again resulted in augmented DNA synthesis as assessed by ^{3}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 upon 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 anti-proliferative 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.

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 up-regulated 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 CCR) and BC patients (66 patients in total). The samples were provided by two different hospitals and their relevant clinicopathological features are shown in Table 1. MYC mRNA levels were determined by qRT-PCR and the results showed that MYC expression was 2-5-fold higher in patients at diagnosis and non-responders 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 the expression of MYC in the same 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 out 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 out of 15) during CML progression. Some representative cases are shown in Fig. 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, 54). 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 CMR. Although the number of samples analyzed was small, the results demonstrate 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 out of 12 samples where mRNA could be analysed (not shown). Thus, the results indicate the increased MYC expression in CML occurs also at the protein level

MYC mRNA levels are higher in non-responders.

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% Confidence Interval (CI) = 0.87 - 0.97. The relationship between MYC levels and 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 non-responders 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 non-responder patients we compared ROC curves with the data of MYC at diagnosis and MYC after treatment in responders and non-responders. 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 panel), whereas it did not mark any difference between patients at diagnosis and non-responders (Fig. 5C left panel). As a control, expression of BCR-ABL was also plotted. The analysis of the ROC curves allows the selection of a MYC cut-off value of 0.058 with a sensitivity of 0.89 and specificity of 0.65. Remarkably, this cut-off is consistent with the mean MYC value of healthy controls and responders (Fig. 4A).

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

Cell culture data indicated that BCR-ABL kinase activity induces MYC and imatinib down-regulates 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 upon 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 hematological response 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 non-responders and blastic crisis 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 hematological response.

Discussion

In this work we describe four 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 two 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 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 (15)].

We detected a 2 to 5-fold increase in MYC expression in patients at diagnosis as compared to healthy controls 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 two-fold change means a major difference for MYC ability to transform cells in different cell culture models (55-57) as well as in transgenic animals where MYC dosage can be modulated (58). It is also noteworthy that in Burkitt lymphoma, the paradigm of MYC activation in human cancer, MYC increase in expression can be only two-fold with respect to normal lymphocytes (59). It is surprising that MYC involvement in CML has gone unnoticed up to now, although the microarray expression data of a previous study shows increased MYC expression in CD34+ cells from 9 CML patients, as compared to healthy controls (39). Other microarray studies have detected upregulation of MYC downstream genes (60-62). A report

described similar expression changes in CML progression for BMI1 (63), a polycomb group gene that cooperates with MYC in carcinogenesis (64), 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 (65). Also, infection of murine bone marrow with MYC retroviruses results in myeloid but not lymphoid leukemia (66, 67). Moreover, analysis in transgenic models shows that MYC is essential for normal differentiation of myeloid stem cells [reviewed in (68)].

How could MYC contribute to CML progression? Our data showing MYC overexpression during CML progression is consistent with our studies in CML blast crisis cells, where MYC promotes aberrant DNA synthesis under imatinib stress. CML progression is associated to increased genomic instability and differentiation arrest, two major and well-known MYC activities [reviewed in (6, 7, 69)]. 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.

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 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^{TM} plasmid. This vector expresses the Myc-ER chimera, which is activated by 4-hydroxytamoxifen (4HT) (45). Transfected clones were selected with 1 μg/ml of puromycin. KLBcl2v cells are K562 cells expressing Bcl2 (53). To generate the KmycBcl2 cell line, KmycB cells were retrovirally transduced with a Bcl2 expression vector as described (53). To generate the KmycBT315I cell line, KmycB cells were electroporated with an expression vector for BCR-ABL-T315I mutant (pSRαp210T315I) (70), All cell lines were grown in RPMI 1640 medium (Gibco-Life

Sciences) containing 10% fetal calf serum, 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 per ml on day 0. For thymidine incorporation assays, cells were incubated with 1 µCi/ml of ^{3}H-thymidine for 2 h, 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 µM bromodeoxyuridine (BrdU) for 90 min and processed as described (71).

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 min and then analyzed by flow cytometry using CellQuest software. For double labeling with BrdU and PI, the cells were pulsed with 10 µM BrdU for 45 min, harvested, fixed in 75% ethanol at 4ºC, washed with PBS and incubated for 20 min with 2 N HCl, neutralized with 0.1 M 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 min at RT washed, and incubated for 60 min with a FITC-conjugated secondary antibody (1:100, Jackson Immuno). Cells were washed, and incubated for 2 h 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 (43).

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 (71). 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 [α-^{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 RT-PCR. Reverse transcription and quantitative PCR were performed as described (72). MYC expression was normalized against the mRNA levels of ribosomal protein S14 (RPS14). The primers for MYC were 5'-AAGACTCCAGCGCCTTCTCTC-3' and 5'-GTTTTCCAACTCCGGGATCTG-3'. The primers for RPS14 were 5'-TCACCGCCCTACACATCAAACT-3' and 5'-CTGCGAGTGCTGTCAGAGG-3'. RPS14 has not been described as a MYC target gene (www.myccancergene.org). Primers for BCR-ABL were as described (73). The experimental variability was controlled using a control cDNA pool synthesized with RNAs from K562 and HeLa cells (50% each).

Luciferase reporter assays

3 million KMER4 cells were electroporated at 260 V and 1 mFa in a Bio-Rad electroporator with 3 µg of pGL2-M4-Luc reporter, which carries four E-boxes in the promoter (74) and 1 µg of the *Renilla* luciferase vector pRL-TK (Promega). After 24 h of incubation, cultures were split into aliquots and further incubated for 24 h with 200 nM 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 two 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 hematological (HR), complete cytogenetic (CCR), major molecular (MMR) and complete molecular (CMR) responses and classified into optimal and suboptimal responses as described (75). Treatment failure was also recorded in nine patients. For our statistical analyses we have considered as non-responder 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 two 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 cut-off. Patients with lack of response at the end 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 $p(response) = \frac{e^{OR}}{1+e^{OR}}$ (76). The efficacy of MYC expression to classify patients into responders and non-responders was studied by generating receiver operating characteristic (ROC) curves. MYC and BCR-ABL expression were compared by Sperman's Rho and Pearson´s Correlation Coefficient. All p-values were calculated from two- side tests and values below 0.05 were considered significant.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no potential conflicts of interest

References

1. Dang CV, O'Donnell K A, Zeller KI, Nguyen T, Osthus RC, Li F. The c-Myc target gene network. Semin Cancer Biol 2006;16:253-64.
2. Eilers M, Eisenman RN. Myc's broad reach. Genes Dev 2008;22:2755-66.
3. Nesbit CE, Tersak JM, Prochownik EV. MYC oncogenes and human neoplastic disease. Oncogene 1999;18:3004-16.
4. Vita M, Henriksson M. The Myc oncoprotein as a therapeutic target for human cancer. Semin Cancer Biol 2006;16:318-30.
5. Meyer N, Penn LZ. Reflecting on 25 years with MYC. Nat Rev Cancer 2008;8:976-90.
6. Wade M, Wahl GM. c-Myc, genome instability, and tumorigenesis: the devil is in the details. Curr Top Microbiol Immunol 2006;302:169-203.
7. Prochownik EV, Li Y. The ever expanding role for c-Myc in promoting genomic instability. Cell Cycle 2007;6:1024-9.
8. Lutz W, Leon J, Eilers M. Contributions of Myc to tumorigenesis. Biochim Biophys Acta 2002;1602:61-71.

9. Karlsson A, Deb-Basu D, Cherry A, Turner S, Ford J, Felsher DW. Defective double-strand DNA break repair and chromosomal translocations by MYC overexpression. Proc Natl Acad Sci U S A 2003;100:9974-9.
10. Li Q, Dang CV. c-Myc overexpression uncouples DNA replication from mitosis. Mol Cell Biol 1999;19:5339-51.
11. Kuschak TI, Kuschak BC, Taylor CL, Wright JA, Wiener F, Mai S. c-Myc initiates illegitimate replication of the ribonucleotide reductase R2 gene. Oncogene 2002;21:909-20.
12. Sheen JH, Dickson RB. Overexpression of c-Myc alters G(1)/S arrest following ionizing radiation. Mol Cell Biol 2002;22:1819-33.
13. Dominguez-Sola D, Ying CY, Grandori C, et al. Non-transcriptional control of DNA replication by c-Myc. Nature 2007;448:445-51.
14. Robinson K, Asawachaicharn N, Galloway DA, Grandori C. c-Myc accelerates S-Phase and requires WRN to avoid replication stress. PLoS One 2009;4:e5951.
15. Herold S, Herkert B, Eilers M. Facilitating replication under stress: an oncogenic function of MYC? Nat Rev Cancer 2009;9:441-4.
16. Felsher DW, Zetterberg A, Zhu J, Tlsty T, Bishop JM. Overexpression of MYC causes p53-dependent G2 arrest of normal fibroblasts. Proc Natl Acad Sci U S A 2000;97:10544-8.
17. Cowling VH, Chandriani S, Whitfield ML, Cole MD. A conserved Myc protein domain, MBIV, regulates DNA binding, apoptosis, transformation, and G2 arrest. Mol Cell Biol 2006;26:4226-39.
18. Gatti G, Maresca G, Natoli M, et al. MYC prevents apoptosis and enhances endoreduplication induced by paclitaxel. PLoS One 2009;4:e5442.
19. Faderl S, Talpaz M, Estrov Z, O'Brien S, Kurzrock R, Kantarjian HM. The biology of chronic myeloid leukemia. N Engl J Med 1999;341:164-72.
20. O'Dwyer ME, Mauro MJ, Druker BJ. Recent advancements in the treatment of chronic myelogenous leukemia. Annu Rev Med 2002;53:369-81.
21. Ren R. Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia. Nat Rev Cancer 2005;5:172-83.
22. Quintas-Cardama A, Cortes J. Molecular biology of bcr-abl1-positive chronic myeloid leukemia. Blood 2009;113:1619-30.
23. Clark RE. Facts and uncertainties in monitoring treatment response in chronic myeloid leukaemia. Leuk Res 2009;33:1151-5.
24. Baccarani M, Pane F, Saglio G. Monitoring treatment of chronic myeloid leukemia. Haematologica 2008;93:161-9.

25. Melo JV, Barnes DJ. Chronic myeloid leukaemia as a model of disease evolution in human cancer. Nat Rev Cancer 2007;7:441-53.
26. Savona M, Talpaz M. Getting to the stem of chronic myeloid leukaemia. Nat Rev Cancer 2008;8:341-50.
27. Kavalerchik E, Goff D, Jamieson CH. Chronic myeloid leukemia stem cells. J Clin Oncol 2008;26:2911-5.
28. Khorashad JS, Wagner S, Greener L, et al. The level of BCR-ABL1 kinase activity before treatment does not identify chronic myeloid leukemia patients who fail to achieve a complete cytogenetic response on imatinib. Haematologica 2009;94:861-4.
29. Xie S, Lin H, Sun T, Arlinghaus RB. Jak2 is involved in c-Myc induction by Bcr-Abl. Oncogene 2002;21:7137-46.
30. Gomez-Casares MT, Vaque JP, Lemes A, Molero T, Delgado MD, Leon J. C-myc expression in cell lines derived from chronic myeloid leukemia. Haematologica 2004;89:241-3.
31. Samanta AK, Lin H, Sun T, Kantarjian H, Arlinghaus RB. Janus kinase 2: a critical target in chronic myelogenous leukemia. Cancer Res 2006;66:6468-72.
32. Lugo TG, Witte ON. The BCR-ABL oncogene transforms Rat-1 cells and cooperates with v-myc. Mol Cell Biol 1989;9:1263-70.
33. Sawyers CL, Callahan W, Witte ON. Dominant negative MYC blocks transformation by ABL oncogenes. Cell 1992;70:901-10.
34. Afar DE, Goga A, McLaughlin J, Witte ON, Sawyers CL. Differential complementation of Bcr-Abl point mutants with c-Myc. Science 1994;264:424-6.
35. Handa H, Hegde UP, Kotelnikov VM, et al. Bcl-2 and c-myc expression, cell cycle kinetics and apoptosis during the progression of chronic myelogenous leukemia from diagnosis to blastic phase. Leuk Res 1997;21:479-89.
36. Beck Z, Bacsi A, Kovacs E, et al. Changes in oncogene expression implicated in evolution of chronic granulocytic leukemia from its chronic phase to acceleration. Leuk Lymphoma 1998;30:293-306.
37. Notari M, Neviani P, Santhanam R, et al. A MAPK/HNRPK pathway controls BCR/ABL oncogenic potential by regulating MYC mRNA translation. Blood 2006;107:2507-16.
38. Blick M, Romero P, Talpaz M, et al. Molecular characteristics of chronic myelogenous leukemia in blast crisis. Cancer Genet Cytogenet 1987;27:349-56.

39. Diaz-Blanco E, Bruns I, Neumann F, et al. Molecular signature of CD34(+) hematopoietic stem and progenitor cells of patients with CML in chronic phase. Leukemia 2007;21:494-504.
40. Johansson B, Fioretos T, Mitelman F. Cytogenetic and molecular genetic evolution of chronic myeloid leukemia. Acta Haematol 2002;107:76-94.
41. Brazma D, Grace C, Howard J, et al. Genomic profile of chronic myelogenous leukemia: Imbalances associated with disease progression. Genes Chromosomes Cancer 2007;46:1039-50.
42. Delgado MD, Lerga A, Canelles M, Gomez-Casares MT, Leon J. Differential regulation of Max and role of c-Myc during erythroid and myelomonocytic differentiation of K562 cells. Oncogene 1995;10:1659-65.
43. Canelles M, Delgado MD, Hyland KM, et al. Max and inhibitory c-Myc mutants induce erythroid differentiation and resistance to apoptosis in human myeloid leukemia cells. Oncogene 1997;14:1315-27.
44. Munoz-Alonso MJ, Acosta JC, Richard C, Delgado MD, Sedivy J, Leon J. p21Cip1 and p27Kip1 induce distinct cell cycle effects and differentiation programs in myeloid leukemia cells. J Biol Chem 2005;280:18120-9.
45. Littlewood TD, Hancock DC, Danielian PS, Parker MG, Evan GI. A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nucleic Acids Res 1995;23:1686-90.
46. Penn LJ, Brooks MW, Laufer EM, Land H. Negative autoregulation of c-myc transcription. Embo J 1990;9:1113-21.
47. Yin XY, Grove L, Datta NS, Long MW, Prochownik EV. C-myc overexpression and p53 loss cooperate to promote genomic instability. Oncogene 1999;18:1177-84.
48. Vafa O, Wade M, Kern S, et al. c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability. Mol Cell 2002;9:1031-44.
49. Ceballos E, Munoz-Alonso MJ, Berwanger B, et al. Inhibitory effect of c-Myc on p53-induced apoptosis in leukemia cells. Microarray analysis reveals defective induction of p53 target genes and upregulation of chaperone genes. Oncogene 2005;24:4559-71.
50. Ceballos E, Delgado MD, Gutierrez P, et al. c-Myc antagonizes the effect of p53 on apoptosis and p21WAF1 transactivation in K562 leukemia cells. Oncogene 2000;19:2194-204.
51. Gorre ME, Sawyers CL. Molecular mechanisms of resistance to STI571 in chronic myeloid leukemia. Curr Opin Hematol 2002;9:303-7.

52. Wu J, Meng F, Kong LY, et al. Association between imatinib-resistant BCR-ABL mutation-negative leukemia and persistent activation of LYN kinase. J Natl Cancer Inst 2008;100:926-39.
53. Lerga A, Richard C, Delgado MD, et al. Apoptosis and mitotic arrest are two independent effects of the protein phosphatases inhibitor okadaic acid in K562 leukemia cells. Biochem Biophys Res Commun 1999;260:256-64.
54. Sears RC. The life cycle of C-myc: from synthesis to degradation. Cell Cycle 2004;3:1133-7.
55. Lee WM, Schwab M, Westaway D, Varmus HE. Augmented expression of normal c-myc is sufficient for cotransformation of rat embryo cells with a mutant ras gene. Mol Cell Biol 1985;5:3345-56.
56. Bazarov AV, Adachi S, Li SF, Mateyak MK, Wei S, Sedivy JM. A modest reduction in c-myc expression has minimal effects on cell growth and apoptosis but dramatically reduces susceptibility to Ras and Raf transformation. Cancer Res 2001;61:1178-86.
57. Baudino TA, McKay C, Pendeville-Samain H, et al. c-Myc is essential for vasculogenesis and angiogenesis during development and tumor progression. Genes Dev 2002;16:2530-43.
58. Murphy DJ, Junttila MR, Pouyet L, et al. Distinct thresholds govern Myc's biological output in vivo. Cancer Cell 2008;14:447-57.
59. Wilda M, Busch K, Klose I, et al. Level of MYC overexpression in pediatric Burkitt's lymphoma is strongly dependent on genomic breakpoint location within the MYC locus. Genes Chromosomes Cancer 2004;41:178-82.
60. Nowicki MO, Pawlowski P, Fischer T, Hess G, Pawlowski T, Skorski T. Chronic myelogenous leukemia molecular signature. Oncogene 2003;22:3952-63.
61. McLean LA, Gathmann I, Capdeville R, Polymeropoulos MH, Dressman M. Pharmacogenomic analysis of cytogenetic response in chronic myeloid leukemia patients treated with imatinib. Clin Cancer Res 2004;10:155-65.
62. Oehler VG, Yeung KY, Choi YE, Bumgarner RE, Raftery AE, Radich JP. The derivation of diagnostic markers of chronic myeloid leukemia progression from microarray data. Blood 2009.
63. Mohty M, Yong AS, Szydlo RM, Apperley JF, Melo JV. The polycomb group BMI1 gene is a molecular marker for predicting prognosis of chronic myeloid leukemia. Blood 2007;110:380-3.
64. Guney I, Sedivy JM. Cellular senescence, epigenetic switches and c-Myc. Cell Cycle 2006;5:2319-23.

65. Smith DP, Bath ML, Metcalf D, Harris AW, Cory S. MYC levels govern hematopoietic tumor type and latency in transgenic mice. Blood 2006;108:653-61.
66. Luo H, Li Q, O'Neal J, Kreisel F, Le Beau MM, Tomasson MH. c-Myc rapidly induces acute myeloid leukemia in mice without evidence of lymphoma-associated antiapoptotic mutations. Blood 2005;106:2452-61.
67. Beverly LJ, Varmus HE. MYC-induced myeloid leukemogenesis is accelerated by all six members of the antiapoptotic BCL family. Oncogene 2009;28:1274-9.
68. Delgado MD, Leon J. Myc roles in hematopoiesis and leukemia. Genes Cancer 2010;1:605-16.
69. Leon J, Ferrandiz N, Acosta JC, Delgado MD. Inhibition of cell differentiation: A critical mechanism for MYC-mediated carcinogenesis? Cell Cycle 2009;8:1148-57.
70. Griswold IJ, MacPartlin M, Bumm T, et al. Kinase domain mutants of Bcr-Abl exhibit altered transformation potency, kinase activity, and substrate utilization, irrespective of sensitivity to imatinib. Mol Cell Biol 2006;26:6082-93.
71. Acosta JC, Ferrandiz N, Bretones G, et al. Myc inhibits p27-induced erythroid differentiation of leukemia cells by repressing erythroid master genes without reversing p27-mediated cell cycle arrest. Mol Cell Biol 2008;28:7286-95.
72. Albajar M, Gutierrez P, Richard C, et al. PU.1 expression is restored upon treatment of chronic myeloid leukemia patients. Cancer Lett 2008;270:328-36.
73. Gabert J, Beillard E, van der Velden VH, et al. Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia - a Europe Against Cancer program. Leukemia 2003;17:2318-57.
74. Hurlin PJ, Queva C, Eisenman RN. Mnt, a novel Max-interacting protein is coexpressed with Myc in proliferating cells and mediates repression at Myc binding sites. Genes Dev 1997;11:44-58.
75. Baccarani M, Cortes J, Pane F, et al. Chronic myeloid leukemia: an update of concepts and management recommendations of European LeukemiaNet. J Clin Oncol 2009;27:6041-51.
76. Neuhaus JM. Statistical methods for longitudinal and clustered designs with binary responses. Stat Methods Med Res 1992;1:249-73.
77. Ferrandiz N, Caraballo JM, Albajar M, et al. p21(Cip1) confers resistance to imatinib in human chronic myeloid leukemia cells. Cancer Lett 2010;292:133-9.

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Figure legends

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 μM imatinib and 75 μM $ZnSO_4$ for 24, 48 or 72 h 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 h. Protein extracts were analyzed by immunoblotting with antibodies to MYC and α-tubulin as a loading control. **C**. Cell proliferation of KmycB cells treated with 0.5 μM imatinib and 75 μM $ZnSO_4$ as in A. **D.** DNA synthesis measured by thymidine (Thy) incorporation. Cells were treated for 72 h with imatinib and DNA synthesis was determined by ^{3}H-thymidine incorporation. Data are mean values from four 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 μM imatinib and 75 μM $ZnSO_4$ for 72 h. Data are mean values from four experiments. **F.** MYC induces an accumulation of cells with 4C DNA content in the presence of imatinib. KmycB cells were treated for 48 h with 0.5 μM imatinib and 75 μM $ZnSO_4$ and the fraction of live cells in each cell cycle phase was determined by propidium iodide staining. The fraction of cells with 4C DNA content is indicated in each case. The data are mean values from five independent experiments. Bars indicate S.E.M.

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 h

with 0.5 μM imatinib and 200 nM 4HT. The levels of the fusion protein MycER and the endogenous MYC were determined by immunoblot. Levels of α-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 four MYC-responsive elements (pGL2-M4-luc) upstream of the firefly luciferase gene. 24h after transfection, cells were treated with 200 nM 4HT for an additional 24 h and the luciferase activity determined, normalized to the *Renilla* luciferase internal control. Data represent the mean of three independent experiments; bars indicate SEM. **C**. Cell proliferation assay of KMER4 cells treated with 0.5 μM imatinib and 200 nM 4HT for 72 h. **D.** DNA synthesis measured by thymidine (Thy) incorporation. Cells were treated for 72 h with imatinib and DNA synthesis was determined by ^{3}H-thymidine incorporation. Data are mean values from three 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 μM imatinib and 200 nM 4HT for 72 h. Data are mean values from three experiments. **F**. MYC induces an accumulation of cells with 4C DNA content in the presence of imatinib. KMER4 cells were treated for 48 h with 0.5 μM imatinib and 200 nM 4HT and the fraction of live cells in each cell cycle phase was determined by propidium iodide staining. The fraction of cells with 4C DNA content is indicated in each case. The data are mean values from three independent experiments. **G**. MYC induces DNA synthesis in 4C DNA cells treated with imatinib. Double labeling with BrdU and propidium iodide (PI) showing DNA synthesis in G2 cells. KMER4 cells were treated for 48 h with 0.5 μM imatinib and 200 nM 4HT (KMER4 cells). The percentage of cells with BrdU incorporated (above unlabelled controls) is indicated in each case.

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 μM imatinib and 75 μM ZnSO4 for 48 h and the fraction cells with sub-G1 DNA content was determined by PI staining and flow cytometry. The data are mean values from three 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 three independent experiments. Bars indicate SEM. **C**. Internucleosomal DNA fragmentation assay for KmycB cells treated with 0.5 μM imatinib (Imat.) and 75 μM ZnSO4 for 48 h as indicated. **D.** KmycBcl2 cells were treated for the 48 h with 0.5 μM imatinib and 70 μM ZnSO4 and analysed 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 μM imatinib and 70 μM $ZnSO_4$ as indicated. **F**. Cell proliferation curve showing the partial resistance to imatinib of KmycBcl2. The cells were treated with 70 μM $ZnSO_4$ and 0.5 μM imatinib as indicated and counted up to 72 h. K562 and KLBcl2v were also included as controls. **G.** DNA synthesis measured by thymidine incorporation. Cells were treated for 72 h with imatinib and DNA synthesis was determined by ^{3}H-thymidine incorporation. Data are mean ± SEM from three experiments, and relative to the thymidine incorporation in untreated cells at each time point. The data corresponding to KmycB are the same of Fig. 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 h with 0.5 μM imatinib and 75 μM $ZnSO_4$ and the fraction of live cells in each cell cycle phase was determined by propidium iodide staining. The fraction of cells with 4C DNA content is indicated in each case. The data are mean values from three independent experiments

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. HC, healthy controls; DG, diagnosis; HR, hematological response within the first 3 months of treatment; RE, response to imatinib (at least CCR); NR, no response to imatinib; BC, blastic crisis. n, number of RNA samples. ***, p <0.001. The mean of three replicates are plotted. **B**. MYC expression during CML evolution. The graphs show the expression of MYC and BCR-ABL during treatment of ten representative patients, including four that responded (R) and six patients that did not respond to imatinib (NR). These include two blast crisis (BC). Note that the scales are different. Patient's identification appears in cursive. **C**. Upper panels: Immunoblots showing the MYC protein levels in lysates from bone marrow cells from CML patients at diagnosis (DG) and at CMR as indicated. Lower graph: quantification of the MYC protein signals with respect to ERK2 or total proteins stained with Coomasie Blue (C.B.). The data are mean values and bars indicate S.E.M.

FIGURE 5. MYC levels are higher in non-responder patients. **A**. 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 cut-off value (0.058 with a sensitivity of 0.89 and specificity of 0.65).

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, non-responders, and in blastic crisis. The solid line indicates the linear prediction. Each point represents the mean of two or three mRNA determinations for each gene. Sperman's Rho and Pearson´s Correlation Coefficient are indicated in each case.

Table 1: Clinicopathological characterístics of CML patients

Gender
- Female: 28
- Male: 38

Mean age at diagnosis
- 54 years (range 26-83)

Bcr-Abl transcript
- p210 b3a2: 50
- p210 b2a2: 14
- p210 b2a2 and p190: 2

Stage at the end of the study
- Chronic phase: 57
- Accelerated phase: 0
- Blast crisis: 9

Response to treatment at the end of the study by cytological, 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 (75))
- Too early to evaluate response: 3
- Failure: 9 (8 HR, 1 mCR)
- Suboptimal response: 10 (6 mCR, 1 MCR, 1 CCR, 1 MMR, 1CMR)
- Optimal response: 40 (6 CCR, 21 MMR, 13 CMR)
- No data to evaluate response with these criteria: 4 (1 CCR, 2 MMR, 1CMR)

Treatment
- Only imatinib: 31
- IFN first line + imatinib: 22
- HU/BU first line + imatinib: 13

Abbreviations**:** HR haematological response, mCR minor cytogenetic response, CCR complete cytogenetic response, MMR major molecular response, CMR complete molecular response.

Figure 1. Albajar et al

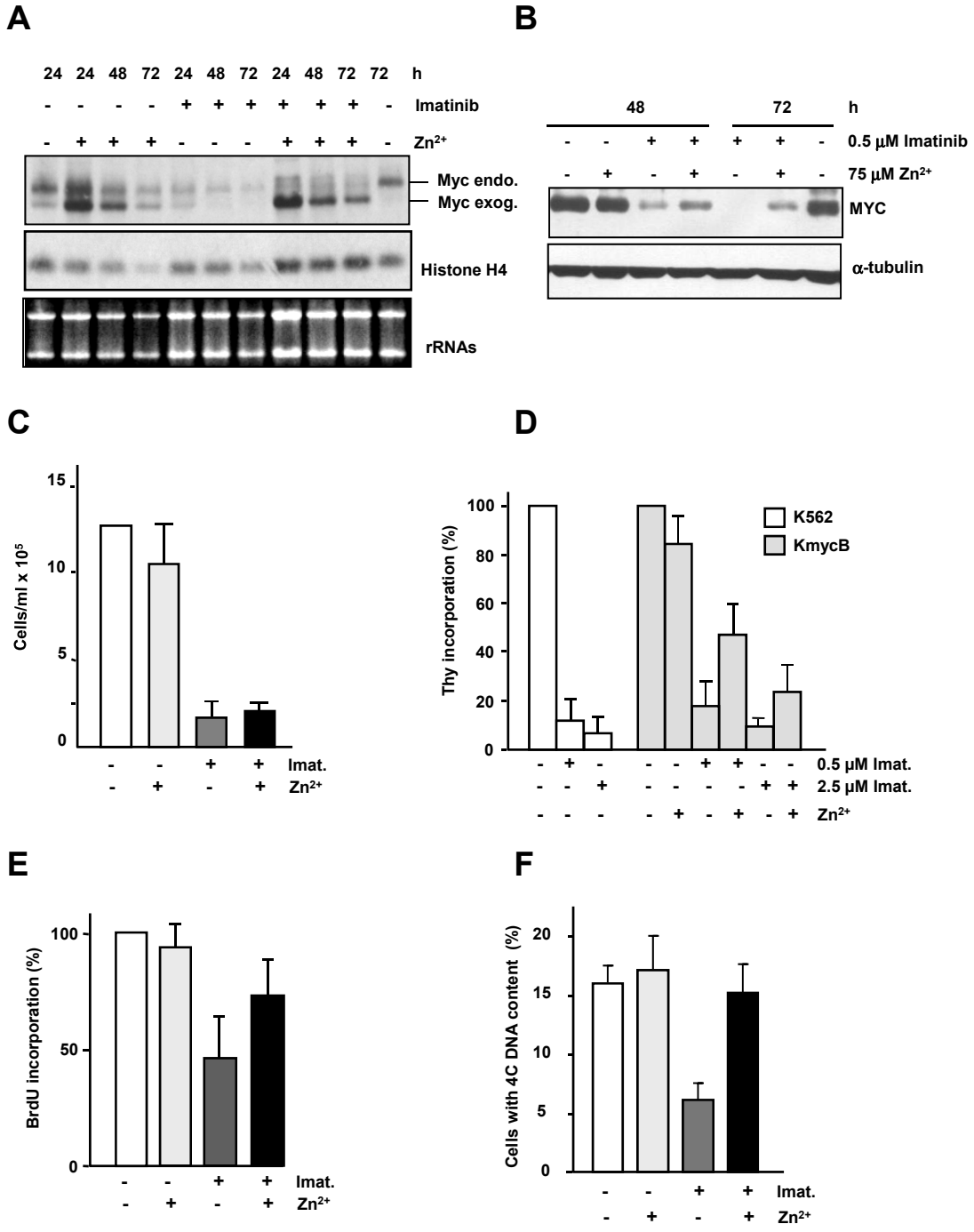


Figure 2. Albajar et al

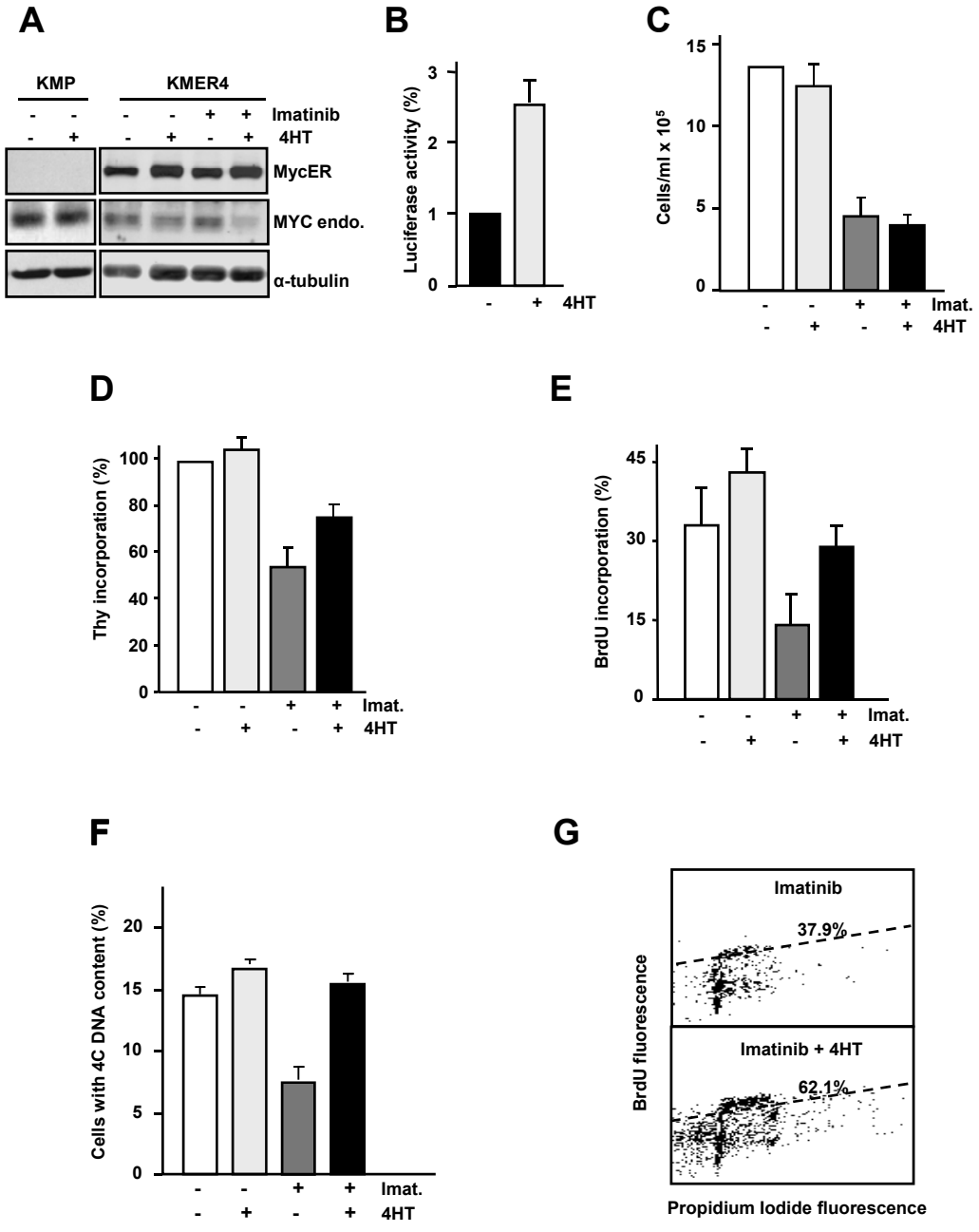
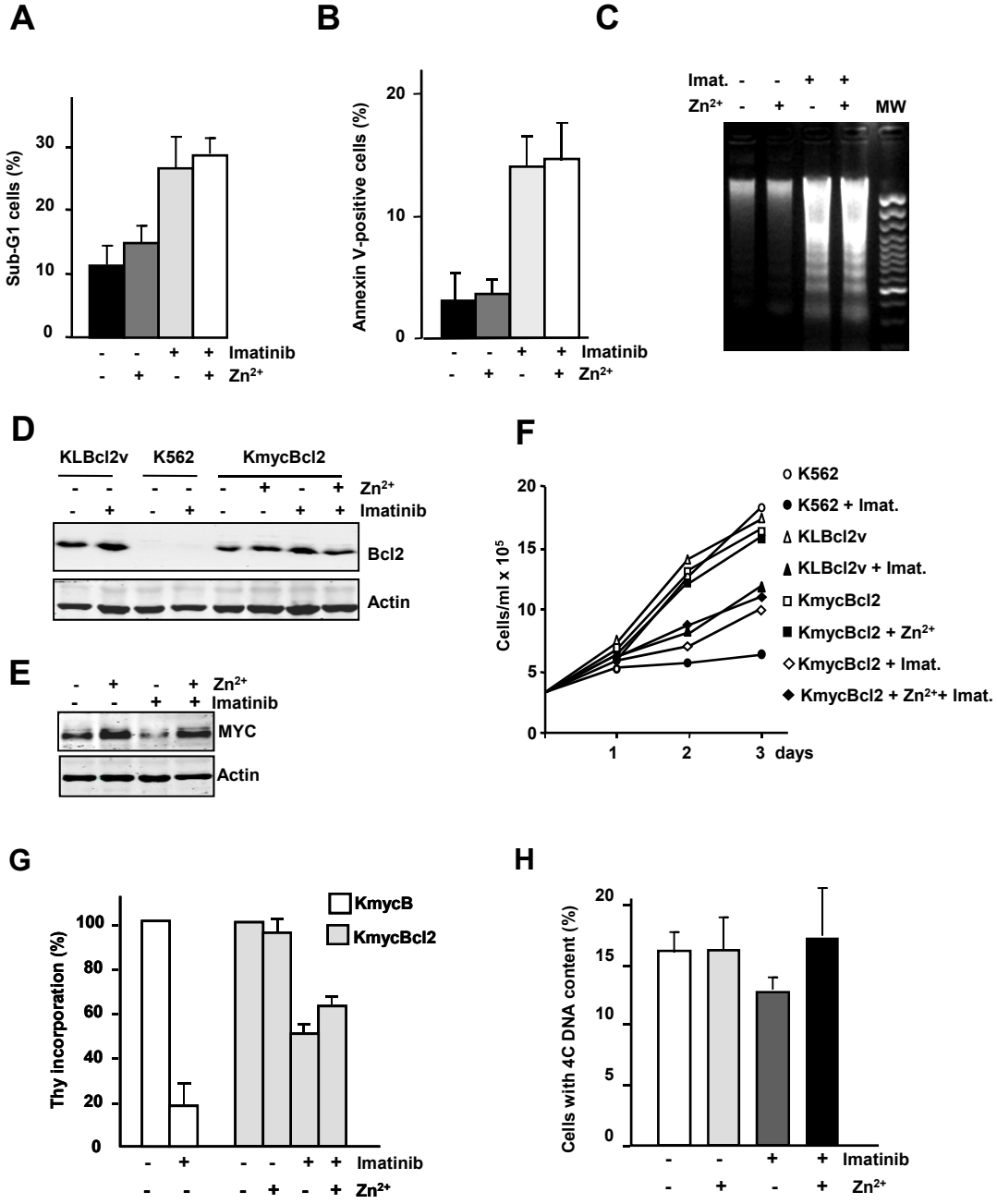


Figure 3. Albajar et al



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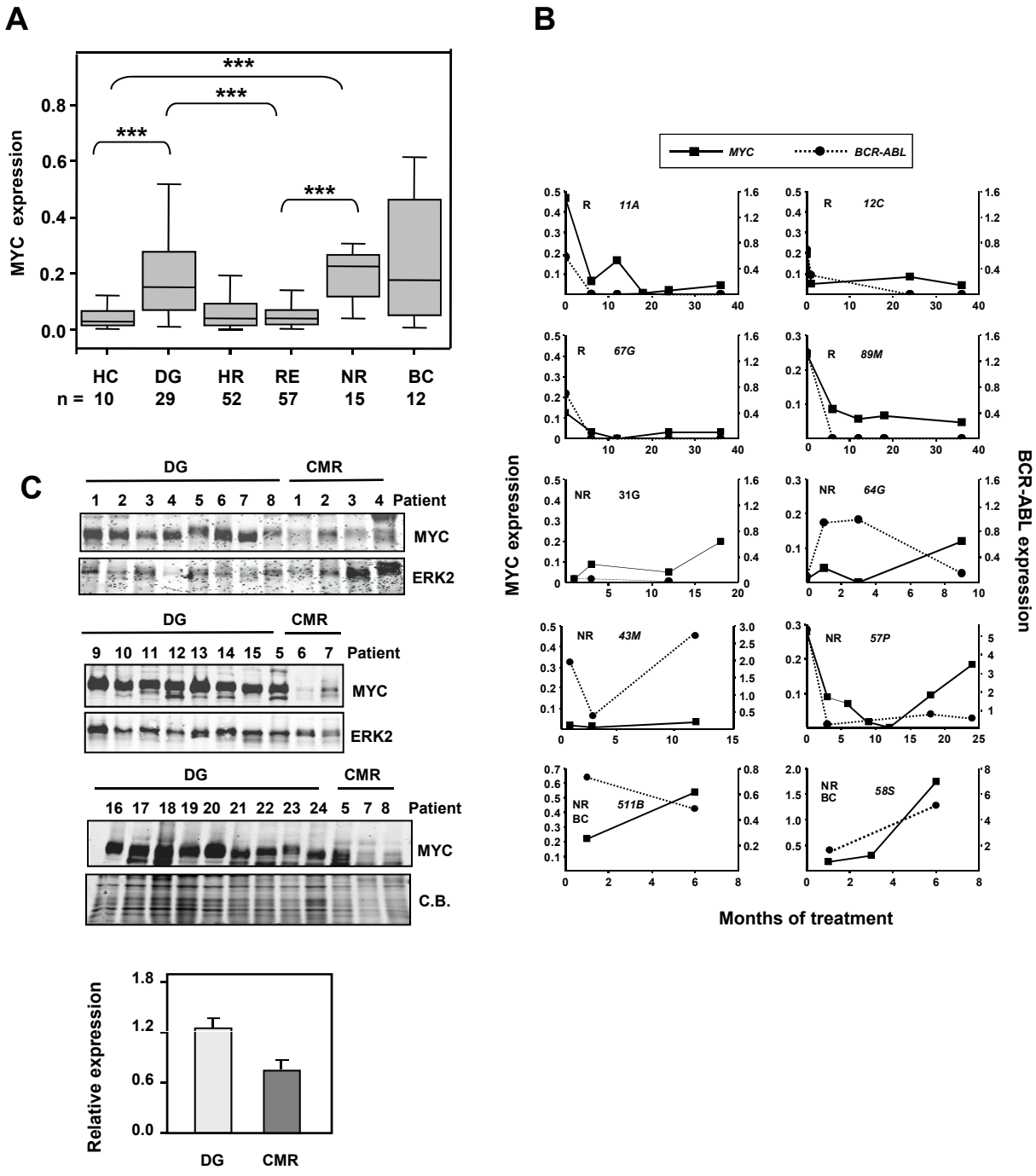

Figure 5. Albajar et al

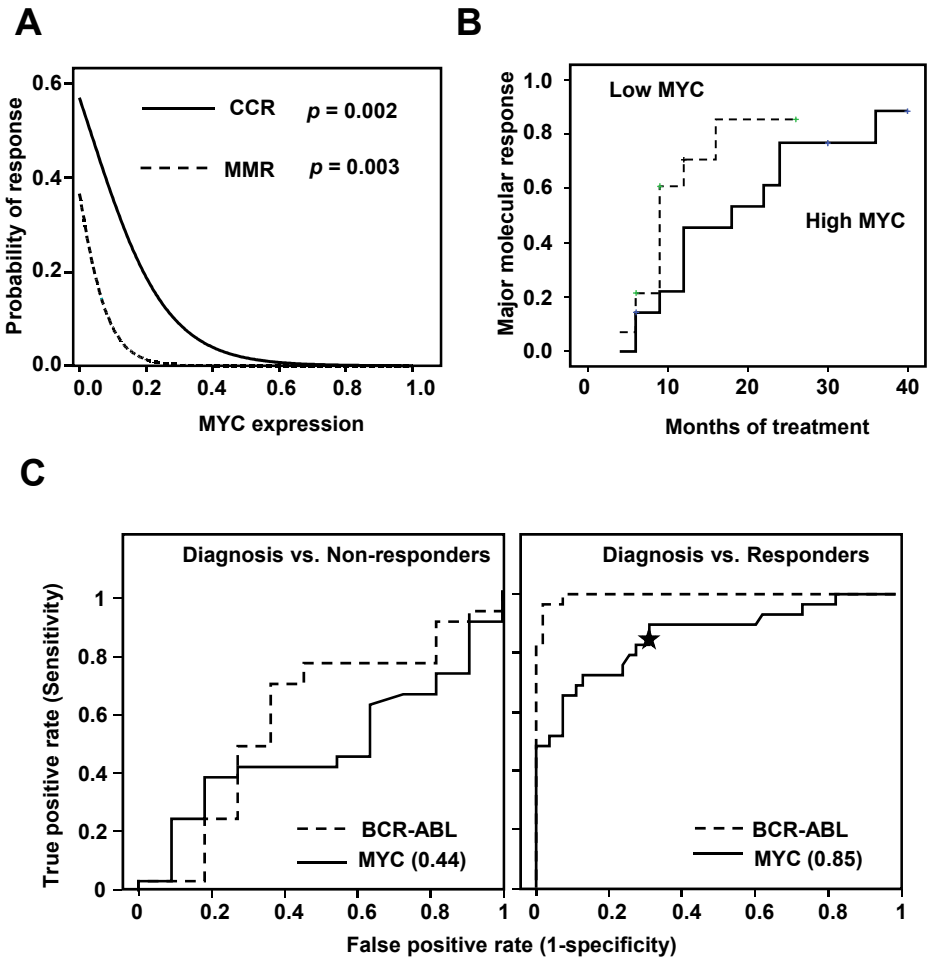

Figure 6. Albajar et al