BCR-ABL1 mediated miR-150 downregulation through MYC contributed to myeloid differentiation block and drug resistance in chronic myeloid leukemia

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ABSTRACT

The fusion oncoprotein BCR-ABL1 exhibits aberrant tyrosine kinase activity and it has been proposed that it deregulates signaling networks involving both transcription factors and non-coding microRNAs that result in chronic myeloid leukemia (CML). Previously, microRNA expression profiling showed deregulated expression of miR-150 and miR-155 in CML. In this study, we placed these findings into the broader context of the MYC/miR-150/MYB/miR-155/PU.1 oncogenic network. We propose that up-regulated MYC and miR-155 in CD34+ leukemic stem and progenitor cells, in concert with BCR-ABL1, impair the molecular mechanisms of myeloid differentiation associated with low miR-150 and PU.1 levels. We revealed that MYC directly occupied the -11.7 kb and -0.35 kb regulatory regions in the *MIR150* gene. MYC occupancy was markedly increased through BCR-ABL1 activity, causing inhibition of *MIR150* gene expression in CML CD34+ and CD34− cells. Furthermore, we found an association between reduced miR-150 levels in CML blast cells and their resistance to tyrosine kinase inhibitors (TKIs). Although TKIs successfully disrupted BCR-ABL1 kinase activity in proliferating CML cells, this treatment did not efficiently target quiescent leukemic stem cells. The study presents new evidence regarding the MYC/miR-150/MYB/miR-155/PU.1 leukemic network established by aberrant BCR-ABL1 activity. The key connecting nodes of this network may serve as potential druggable targets to overcome resistance of CML stem and progenitor cells.

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Introduction

Chronic myeloid leukemia (CML) is a malignant myeloproliferative disease originating from hematopoietic stem cells. The hallmark of CML is the presence of the *BCR-ABL1* fusion gene due to the reciprocal translocation t(9;22)(q34;11). The constitutively active tyrosine kinase activity of the chimeric BCR-ABL1 protein causes deregulation and reprogramming of downstream signaling pathways, and drives the oncogenic process by altering cell proliferation, differentiation and survival. An understanding of CML pathogenesis consequently allowed a rational therapeutic strategy targeting BCR-ABL1 oncoprotein using tyrosine kinase inhibitors (TKIs) to be developed. The introduction of TKIs represented a breakthrough in CML therapy and achieved a large improvement in patient prognosis and outcome, and TKIs became the gold standard for first-line treatment.[1]

Despite the high efficacy of TKIs, 20-30% of CML patients develop resistance during the chronic phase (CP). The frequency of TKI resistance significantly increases as the disease transforms from the CP to fatal blast crisis, which is initially a BCR-ABL1-dependent process;[2] however, an established network further transforms the condition to BCR-ABL1 independence, resulting in a switch to a more aggressive acute leukemia-like disease.[3] Although TKI treatment can successfully ablate the tumor cell population, it does not permanently cure CML because quiescent CML stem cells (LSCs) are often insensitive to TKIs.[4,5] CML LSCs survive and are able to re-initiate the disease after the discontinuation of TKI treatment in some patients.[6]

The dysregulated epigenetic mechanisms previously described in CML involve microRNAs. We and others have shown that miR-150 levels are significantly reduced in CML.[7-10] miR-150 is an inhibitor of the oncogenic transcription factor MYB, which regulates hematopoiesis at the early progenitor levels,[11] while its inappropriate levels during later stages block cell differentiation.[12,13] In a mouse model of CML blast crisis, c-MYB was shown to be required for BCR-ABL1-dependent leukemogenesis.[14] We previously showed that miR-150 and MYB levels are inversely related, and these levels reciprocally respond to TKI treatment.[10] CML in blast crisis shares certain features of acute leukemia. MYB is an upstream factor of acute myeloid leukemia (AML) aggressiveness that positively regulates miR-155. miR-155 inhibits the tumor suppressor and pro-differentiation factor PU.1.[15,16] MYB expression is directly activated by the oncogenic transcription factor MYC in murine virus-induced myeloid leukemia tumor cells.[17] MYC and its partner MAX directly bind the BCR promoter and up-regulate BCR-ABL1 expression.[18]

The functional connections among miR-150, MYC and BCR-ABL1 and the mechanism of the MYB/miR-155/PU.1 network, which is involved in acute leukemogenesis and affects its aggressiveness, led us to evaluate their relationship in CML and TKI resistance.

Methods

Patients' samples

Chronic myeloid leukemia patients were diagnosed and treated at the Institute of Hematology and Blood Transfusion in Prague (UHKT), Czech Republic, and the Marlene and Stuart Greenebaum Comprehensive Cancer Center at the University of Maryland, USA. The bone marrow (BM) samples (n=46) from the CML patients in CP (n=41) and peripheral blood mononuclear cells (PBMCs) samples (n=10) from healthy volunteers were obtained with written informed consent according to the principles of the Declaration of Helsinki and approval by the UHKT Ethics Committee. The samples were collected at time of diagnosis (n=28) and at time of TKI resistance (n=18). The therapeutic response was scored according to the European LeukemiaNet recommendations.[19] The response to first-line treatment was assessed after 12 months of therapy (*Online Supplementary Table S1*). Patients' BM samples were used for FACS sorting, and subsequently to evaluate gene expression (Figure 1A-F and *Online Supplementary Figure S1A and B*) and the correlations among them (*Online Supplementary Figure S1C*).

Additional BM samples (n=6) from CML patients in CP (n=3) or blast crisis (n=3), and the BM samples from healthy donors (n=3), were obtained and handled according to the Ethics Committee of the University of Maryland, USA, and used for miRNA sequencing (Figure 2).

The PBMC samples from CML patients in CP at the time of diagnosis (n=3) and additional PBMC samples of healthy donors (n=3) were handled according to the Ethics Committee of the UHKT. Samples were separated into CD34+ and CD34- cells, and used in the study of MYC binding to *MIR150* regulatory regions by chromatin immunoprecipitation.

Leukemic cell lines

The BCR-ABL1-positive CML cell lines K562, MEG-01 and KCL-22 and the BCR-ABL1-negative AML cell lines HL-60 and KG-1 were obtained from a publicly accessible biological resource center (Leibniz Institute - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH/DSMZ, Braunschweig, Germany). The cell lines were handled and cultivated in appropriate medium according to the recommendations of the supplier. The K562R and KCL-22R cell lines resistant to imatinib were established by gradually exposing naive parental cells to increasing concentrations of imatinib in the medium (See *Online Supplementary Appendix* for details). The leukemic cell lines were used to measure gene expression and for functional experiments.

Statistical analysis

Statistical analyses were performed using Student *t*-test in MS Excel (Microsoft Corporation, Redmond, WA, USA); *$P<0.05$, **$P<0.01$ and ***$P<0.001$. All graphs were generated using MATLAB version R2015b and GraphPad (GraphPad, La Jolla, CA, USA). Correlation coefficients calculated by non-parametric Spearman tests were used to determine the positive and negative correlations. Cloud-based MeV4 software (*Multiple Experiment Viewer; http://mev.tm4.org/*)[20] was used to visualize the correlation data. The combinatory effect of miR-150 overexpression and imatinib treatment on MYB expression was assessed using the Highest Single Agent approach (CIB-HSA).[21]

Details regarding patients and primary cell samples, cell sorting and separation, cell lines, transient transfections, siRNAs design, nucleic acid isolation, RT-qPCR assays, protein isolation and immunoblotting, miRNA RNA sequencing, cell cycle and viability analyses are described in detail in the *Online Supplementary Appendix*.

Results

A putative BCR-ABL1/MYC/miR-150/MYB/miR-155 regulatory pathway is activated in chronic myeloid leukemia

To outline relationships among the studied molecules, gene expression levels were evaluated in primary cells sorted according to CD34 expression (see *Online Supplementary Appendix*) from the BM of CML patients at the time of diagnosis (n=28) and at the time of resistance to TKIs (n=18) (Figure 1). We observed significantly lower miR-150 levels and increased MYC expression in CD34+ CML cells ($P<0.0001$ for miR-150 and MYC) and CD34- CML cells ($P=0.0001$ for miR-150; $P=0.0013$ for MYC) at the time of diagnosis compared with those in the CD34+ and CD34- cells of healthy donors (n=10), respectively. MYB expression was significantly down-regulated in CD34+ CML cells ($P<0.0001$) and significantly up-regulated in CD34- CML cells ($P<0.0001$). miR-155 levels were significantly up-regulated in the CD34+ CML cells at the time of diagnosis ($P=0.0002$), while the expression of the pro-differentiation transcription factor PU.1, which is the

validated target of miR-155 in B cells,[15] was significantly down-regulated (*P*<0.0001) (Figure 1). These data highlight the notable similarity between the expression profiles of CML-CP CD34+ and CD34– cells at the time of diagnosis and TKI resistance, respectively. To obtain detailed information regarding the expression of studied molecules in CML cells, CD34+ and CD34– leukemic cells were further sorted into five distinct subpopulations according to CD38 expression: CD34+CD38–; CD34+CD38+; CD34–CD38+; CD34–CD38+low; and CD34–CD38–. A marked gradual increase in miR-150 levels and a decrease in its target MYB[11] were found both among the sorted subpopulations at the time of diagnosis and at the time of TKI resistance (*Online Supplementary Figure S1A*). The increased levels of miR-150 and decreased levels of MYB coincided with a higher degree of cell differentiation (*Online Supplementary Figure S1B*). Negative correlations between the levels of miR-150 and the levels of oncogenes (BCR-ABL1, MYC and MYB) were observed across all five sorted leukemic BM cell subpopulations (*Online Supplementary Figure S1C*).

To investigate the potential roles of miR-150 and miR-155 in different disease phases, we also measured miR-150 and miR-155 expression in the CD34+CD38– and CD34+CD38+ subpopulations from BM samples of CML patients in CP (n=3) and in blast crisis (n=3). The levels of miR-150 and miR-155 in the corresponding cell populations in the CP were not significantly different from those in blast crisis (Figure 2).

BCR-ABL1 deregulated miR-150 and MYB in CML cells

The expression of the studied molecules was further evaluated in CML (K562, KCL-22 and MEG-01) and AML (KG-1 and HL-60) cell lines to address whether *in vitro* models could be used to study functional relationships between the molecules (*Online Supplementary Figure S2*). In accordance with data from primary CML samples, miR-150 levels were significantly decreased in CD34– CML cell lines compared with the levels in the pool of CD34– cells from healthy donors (n=10) (*P*<0.001), while the miR-150 levels were 5-fold lower in the CML than those in the BCR-ABL1-negative AML cells (*P*<0.01), implying that
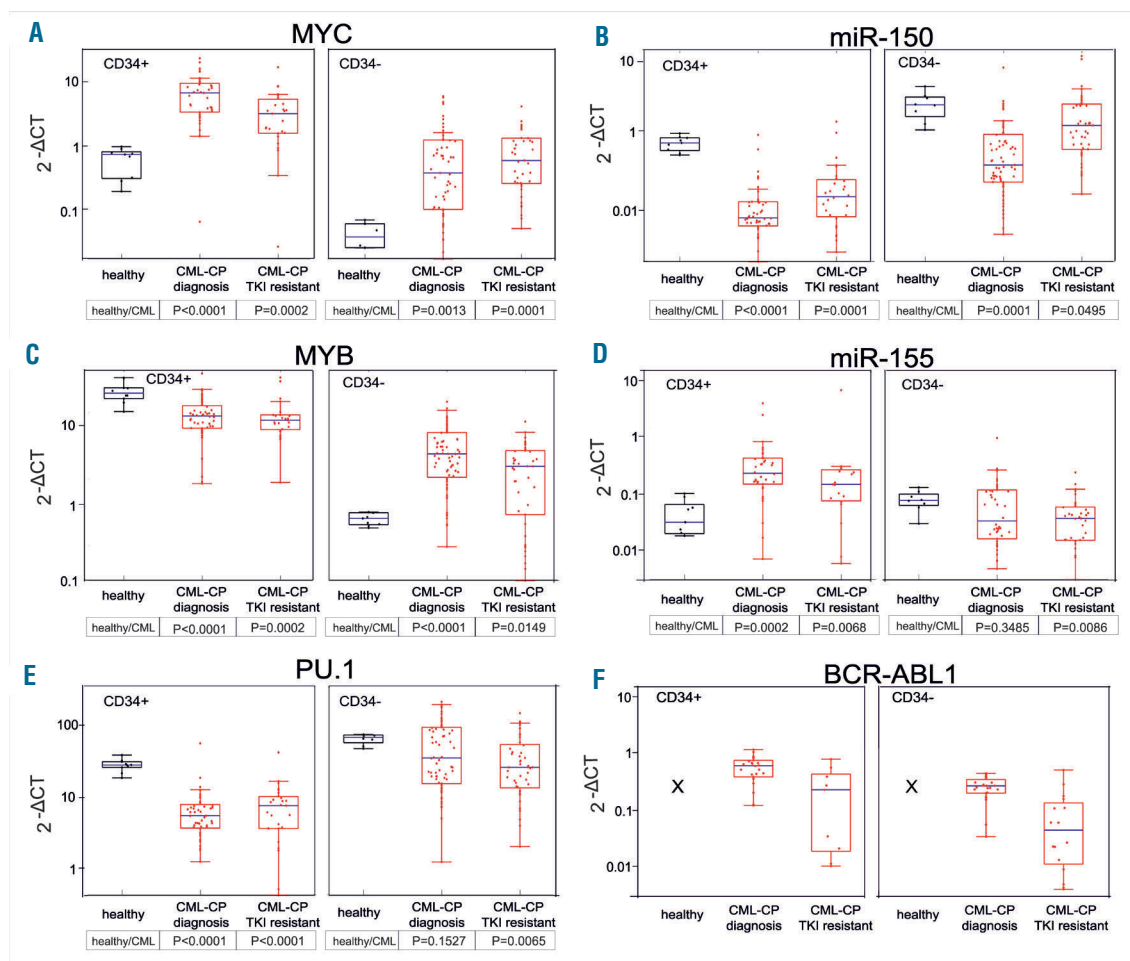
Figure 1. Expression of genes involved in a putative oncogenic pathway in primary chronic myeloid leukemia (CML) and healthy cells. The expression of MYC (A), miR-150 (B), MYB (C), miR-155 (D), PU.1 (E) and BCR-ABL1 (F) in CD34+ (left) and CD34– (right) subpopulations from CML-chronic phase (CP) patients at diagnosis (n=28) or at the time of resistance to tyrosine kinase inhibitors (TKIs) (n=18) compared to that in the CD34+ or CD34– subpopulations of healthy donors (n=10). Gene expression in each specific sample is indicated by dots; the boxes represent box-and-whisker plots. Red: bone marrow (BM) CML-CP samples; black: healthy peripheral blood mononuclear cells (PBMC); blue lines: median expression values. Significant differences (*P*-values) in the gene expression between CML and healthy CD34+ and CD34– subpopulations are illustrated in the tables below each box-and-whisker plot. Unpaired two-tailed Student *t*-test was used to determine *P*-values.

miR-150 expression is likely regulated by BCR-ABL1 activity (*Online Supplementary Figure S2*).

We tested this hypothesis by overexpressing miR-150 (*Online Supplementary Figure S3*) and inhibiting BCR-ABL1 in CML cells. Imatinib (the most frequently used TKI in CML treatment) and/or BCR-ABL1-specific siRNA were used to determine the effect of the BCR-ABL1 activity/expression inhibition.

First, miR-150 overexpression significantly decreased MYB mRNA and protein expression levels in both the BCR-ABL1-positive (K562) and BCR-ABL1-negative (HL-60) cells. MYB protein levels were decreased in KCL-22 cells (Figure 3A-C); the expression of c-MYB (75 KDa) was inhibited in both CML cell lines, while the expression of the spliced 37-KDa MYB variant was affected in HL-60 cells (the 75-KDa MYB variant was not expressed in these cells). MYB expression was down-regulated after the inhibition of BCR-ABL1 activity and expression in the CML cells (Figure 3B-D). Furthermore, simultaneously restoring miR-150 and inhibiting BCR-ABL1 had a positive combination effect on the inhibition of MYB mRNA levels (Highest Single Agent approach value: CI_HSA 0.9654) in

the K562 cells and protein levels in the K562 and KCL-22 cells (Figure 3B and C). There were no significant differences between the effects of imatinib alone or its combination with a miR-150 mimic with regard to the inhibition of MYB expression. Interestingly, miR-155 levels were significantly increased in both K562 and KCL-22 cells after the suppression of BCR-ABL1 activity by imatinib (Figure 3E). The inhibition of BCR-ABL1 activity also significantly decreased PU.1 in K562 cells but significantly increased PU.1 in KCL-22 cells (Figure 3F). This finding is of interest, as K562 cells represent leukemic erythroblasts, whose proper differentiation requires low PU.1 levels, while KCL-22 cells represent leukemic myeloblasts, whose differentiation requires high PU.1 levels.

Next, we found that suppressing BCR-ABL1 activity by imatinib significantly decreased MYC mRNA expression in K562 and KCL-22 cells, while imatinib had no impact on MYC expression in the BCR-ABL1-negative HL-60 cells (Figure 3G). We investigated the possible inhibitory role of BCR-ABL1 and its putative downstream target MYC in miR-150 expression and maturation. The levels of the miR-150 precursors and mature miR-150 consistently increased
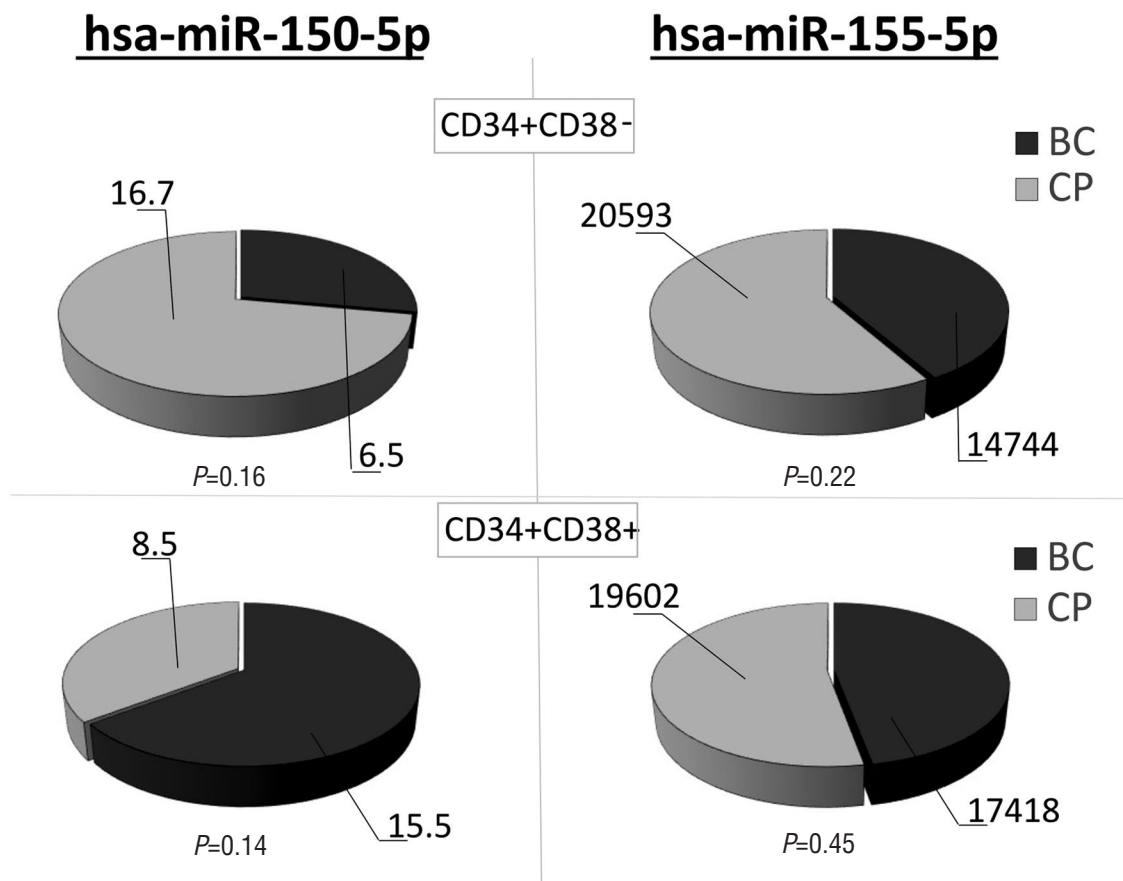

Figure 2. Comparison of miR-150 and miR-155 expression in leukemic stem/progenitor cells (LSPCs) in blast crisis and chronic phase (CP) chronic myeloid leukemia (CML). Pie charts illustrate the proportions of read count median values for miR-150 (left column) and miR-155 (right column) expression obtained by miRNA RNA sequencing. The read counts that overlapped with miR-150 and miR-155 after alignment with human genome GRCh38 were calculated using miRDeep quantifier module and normalized to counts per million (CPM). Primary bone marrow (BM) cell populations used for the measurement and comparison are indicated for each pair of charts. *P*-values in the miR expression between the compared cell populations are illustrated below each chart. Paired two-tailed Student *t*-test was used for determining of *P*-values. BC: blast crisis CML (n=3); CP: chronic phase CML (n=3).

upon the BCR-ABL1 inhibition induced by imatinib in the K562 cells. Thus, BCR-ABL1 inhibits miR-150 transcription without affecting miR-150 maturation (Figure 3H).

The inhibition of BCR-ABL1 using imatinib significantly increased the G0/G1 phase and decreased the S phase in K562 cells, which was accompanied by an increase in apoptosis (*Online Supplementary Figure S4*). Sensitivity to imatinib differed significantly between cell lines, as 50% of the K562 cells but only 5% of the KCL-22 cells underwent apoptosis 96 hours (h) after imatinib treatment

(*Online Supplementary Figure S4B and D*). This difference in imatinib sensitivity had been previously discovered during the development of the resistant cell lines (see *Online Supplementary Appendix*) and is consistent with the ability of KCL-22 cells to continuously grow in the presence of 1 μM imatinib and relapse early due to the acquisition of resistant *BCR-ABL1* mutations or to BCR-ABL1-independent mechanisms.[22] Restoration of miR-150 had no impact on the cell cycle or viability in the K562 cells within 96 h post transfection (*Online Supplementary Figure S4*).
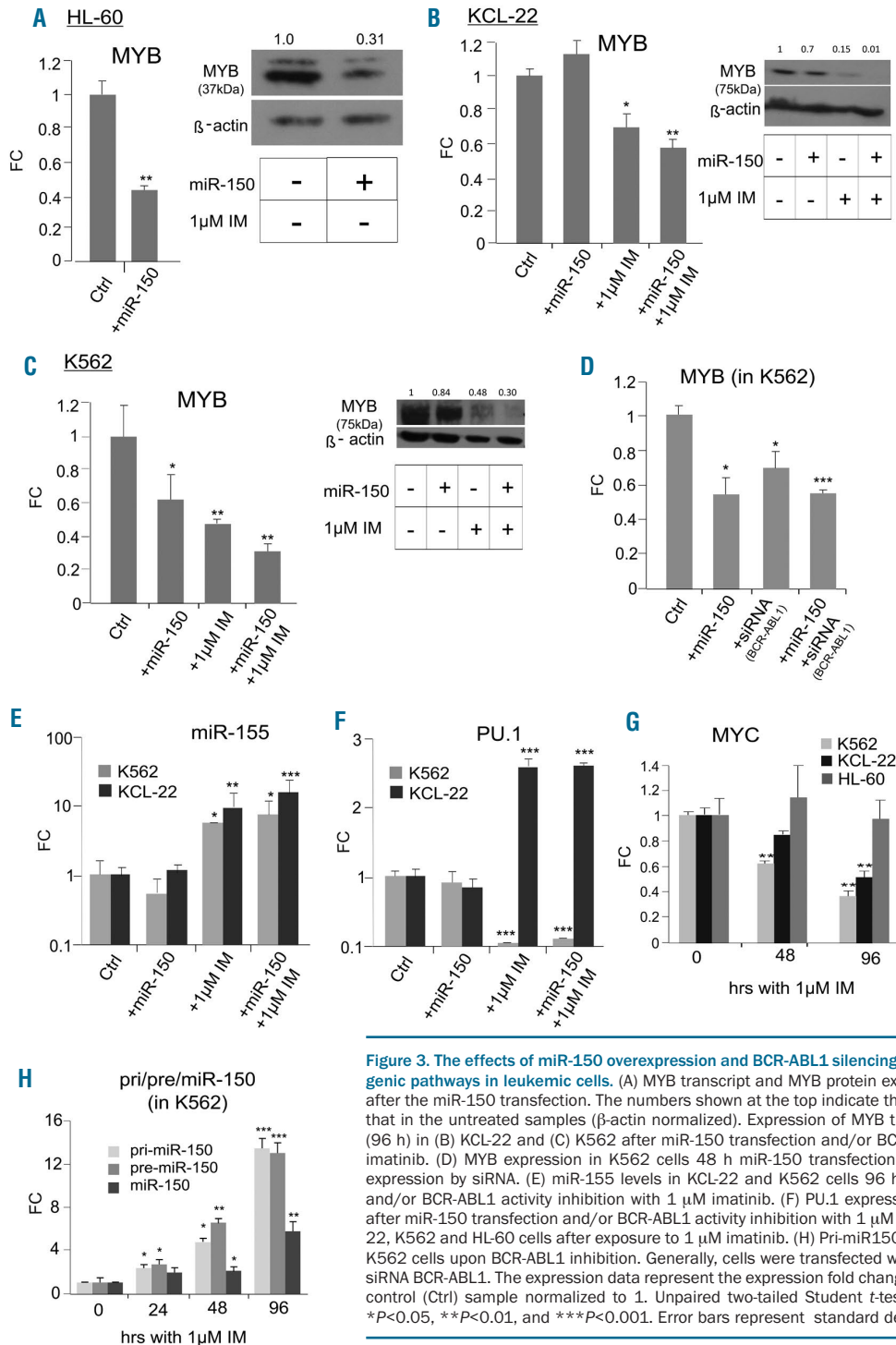
Figure 3. The effects of miR-150 overexpression and BCR-ABL1 silencing on the expression of genes in oncogenic pathways in leukemic cells. (A) MYB transcript and MYB protein expression in HL-60 cells 48 hours (h) after the miR-150 transfection. The numbers shown at the top indicate the MYB protein expression relative to that in the untreated samples (β-actin normalized). Expression of MYB transcripts (48 h) and c-MYB protein (96 h) in (B) KCL-22 and (C) K562 after miR-150 transfection and/or BCR-ABL1 activity inhibition with 1 μM imatinib. (D) MYB expression in K562 cells 48 h miR-150 transfection and/or the inhibition of BCR-ABL1 expression by siRNA. (E) miR-155 levels in KCL-22 and K562 cells 96 h after 30 nM miR-150 transfection and/or BCR-ABL1 activity inhibition with 1 μM imatinib. (F) PU.1 expression in KCL-22 and K562 cells 96 h after miR-150 transfection and/or BCR-ABL1 activity inhibition with 1 μM imatinib. (G) MYC expression in KCL-22, K562 and HL-60 cells after exposure to 1 μM imatinib. (H) Pri-miR150, pre-miR150 and miR-150 levels in K562 cells upon BCR-ABL1 inhibition. Generally, cells were transfected with 30 nM miR-150 mimic or 50 nM siRNA BCR-ABL1. The expression data represent the expression fold change (FC; 2^-ΔΔCt) in relation to untreated, control (Ctrl) sample normalized to 1. Unpaired two-tailed Student *t*-test was used to determine *P*-values. *P<0.05, **P<0.01, and ***P<0.001. Error bars represent standard deviations.

MYC repressed expression of miR-150 in CML cells

Based on the inverse trend of MYC expression and miR-150 levels (*Online Supplementary Figure S1C*), we hypothesized that MYC is directly involved in the repression of *MIR150* gene expression in CML. Publicly available ChIP-Seq data from the UCSC Genome Browser (*http://genome.ucsc.edu/index.html*) were used to predict the protein binding regions surrounding the *MIR150* gene in K562 cells. We identified loci with a high probability of binding to *MIR150* gene regulatory factors such as MYC and its partner MAX (*Online Supplementary Figure S5*). We investigated whether MYC binds these regions in naive and imatinib-treated K562, KCL-22 and HL-60 cells using chromatin immunoprecipitation (ChIP). We observed the MYC occupancy at the loci -11.7 kb and -0.35 kb upstream of the MIR150 gene TSS in untreated K562 and at the loci -11.7 kb in untreated KCL-22 cells, but MYC was not detected at these and other tested loci in the HL-60 cells. The 48-h treatment with imatinib significantly decreased the MYC occupancy in K562 cells and, to a lesser degree, in KCL-22 cells (Figure 4A and B), but the treatment did not impact MYC non-occupancy in HL-60 cells (Figure 4C).

To determine the effect of profound MYC suppression on miR-150 expression, K562 and KCL-22 cells were treated with bromodomain inhibitor JQ1.[23] JQ1 treatment significantly, and to a similar extent, decreased MYC mRNA expression in K562 and KCL-22 cells (*Online Supplementary Figure S6A*), without affecting BCR-ABL1 expression (*Online Supplementary Figure S6B*), and increased miR-150 levels in KCL-22 cells but not in K562 cells (*Online Supplementary Figure S6C*).

Next, we investigated MYC binding to *MIR150* gene regulatory regions in sorted CD34+ and CD34− CML primary cells compared with that in healthy donors. We found marked MYC binding to the -11.7 kb and -0.35 kb loci in CD34+ cells isolated from CML-CP patients (n=3) at the time of diagnosis. In contrast, MYC occupancy at these loci was weak in CD34+ cells from healthy donors (n=3) (Figure 5A). Decreased MYC occupancy to *MIR150* gene regulatory regions was detected in the leukemic CD34− subpopulation, while MYC binding in the tested loci was undetectable in CD34− cells of healthy donors (Figure 5B). These data highlight the role of MYC in regulating the *MIR150* gene in CML.

To investigate a potential regulatory loop between the miR-150 target MYB and different molecules, we investigated the effect of silencing MYB expression using siRNA in K562 and KCL-22 cells. No significant effects on BCR-ABL1, MYC, miR-150, miR-155 and PU.1 levels were observed following near complete MYB inhibition (*Online Supplementary Figure S7*), suggesting that MYB is not involved in the direct regulation of these molecules in CML.

Resistant chronic myeloid leukemia cells further down-regulate miR-150

To address whether the studied leukemic network is involved in TKI resistance in CML, we analyzed gene expression in resistant CML cells using two established distinct models of resistance (see *Online Supplementary Methods*). BCR-ABL1 was strongly over-expressed in all CML cell cultures. Similar BCR-ABL1 transcript levels were observed between the resistant and sensitive parental cell lines (Figure 6). miR-150 and miR-155 levels were significantly lower (*P*<0.001) in both resistant CML cell lines compared with the levels in their respective imatinib-sensitive

counterparts. PU.1 levels were significantly decreased in KCL-22R cells (*P*<0.001) and significantly increased in K562R cells (*P*<0.01) compared with the levels in their sensitive counterparts (Figure 6). These data suggest that further PU.1 deregulation may be associated with the blockade of erythroid (K562) and myeloid (KCL-22) cell differentiation, respectively, in resistant CML.

Model of BCR-ABL1/MYC/miR-150/MYB/miR-155/PU.1 regulatory links in leukemic primary cells

We assessed the BCR-ABL1/MYC/miR-150/MYB/miR-155/PU.1 network of CD34+ and CD34− CML-CP cells at
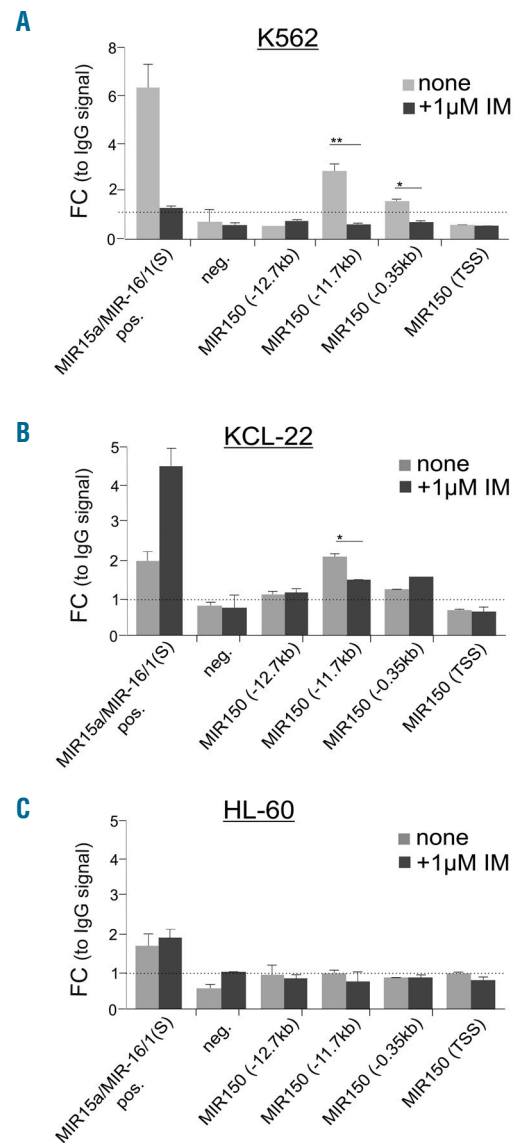
Figure 4. miR-150 regulation by MYC in leukemic blasts. MYC occupancy at putative regulatory loci of the *MIR150* gene in (A) K562, (B) KCL-22 and (C) HL-60 cells with or without imatinib treatment. Columns represent the fold change (FC) in the % of DNA input obtained in the control (gray) or imatinib-treated (dark) cells compared with the non-specific IgG precipitation and equalized to 1 (dashed line). The promoter region of the miR-15a/16-1 cluster was used as the positive control of MYC DNA binding. Unpaired two-tailed Student *t*-test was used to determine *P*-values. *P<0.05, **P<0.01. Error bars represent standard deviations.

the time of diagnosis (Figure 7). The proposed scheme combines the data obtained from expression analyses, the data obtained from functional experiments and chromatin immunoprecipitation assays performed in cell cultures and primary cells to present a simplified model of the regulatory interactions in CML hematopoiesis.

Based on our data and that from the literature, we propose a model illustrating relatively lower levels of MYC and higher levels of miR-150 in normal CD34+ and CD34- cells (Figure 7A and B) compared with those observed in corresponding leukemic cells. As the binding of MYC to the regulatory regions of MIR150 is weak in normal CD34+ cells (i.e. slightly above background) (Figure 5), the model assumes no inhibition of miR-150 expression by MYC in CD34+ cells to ensure the physiological levels of miR-150 required for the early stages of hematopoiesis. MYC transcription has previously been shown to be repressed by PU.1.[24] MYB levels are high in the normal CD34+ cells, suggesting that MYB is required during the early stages of hematopoiesis;[11,12] however, MYB levels are subsequently sharply reduced by abundant miR-150 and, supposedly, transcriptional repression by PU.1[25] in CD34- cells (Figure 7B). The miR-155 levels were lower in healthy CD34+ cells (compared with those in the corresponding leukemic subpopulation), allowing for the proper expression of its targets MYB and PU.1 in healthy hematopoietic stem and progenitor cells. Indeed, PU.1 expression in the normal cells was further increased in the more mature subpopulation, which is consistent with its requirement for proper myeloid lineage development and differentiation.[26] In summary, the model proposes the existence of few 'core' interactions between the studied molecules ensuring the strict regulation of MYC and MYB expression during normal hematopoietic cell differentiation.

In contrast, the suggested model of interactions among the studied molecules in CD34+ and the more differentiated CD34- CML cells (Figure 7C and D) is characterized by the establishment of several aberrant connections caused by BCR-ABL1 activity, resulting in the consistently increased expression of MYC and MYC occupancy in the

MIR150 regulatory regions in CML. As a consequence, the expression levels of miR-150 and PU.1 were lower than those observed in the corresponding cell populations from healthy controls. Due to the repressed expression of miR-150, we would expect MYB overexpression in CML CD34+ cells compared with the expression in normal CD34+ cells, which was not demonstrated. Decreased levels of MYB may be explained by the increased levels of miR-155 targeting MYB in leukemic CD34+ cells.[27] Moreover, miR-155 overexpression in CML CD34+ cells suggests the presence of inhibitory effect on the expression of its target PU.1.[15]

The significantly higher expression of MYC in concert with the BCR-ABL1 activity resulted in downregulation of miR-150 and, consequently, in higher MYB expression levels in CD34- cells compared with the levels in cells from healthy donors.

No marked differences in miR-155 levels were observed between the leukemic and healthy cells in the CD34- subpopulation, and the role of miR-155 is uncertain in these more differentiated cells. The trend for increased PU.1 levels and decreased MYB and BCR-ABL1 levels was found in leukemic CD34- compared with CD34+ cells, corresponding to the fact that the myeloid lineage development and differentiation in CML-CP is not fully arrested (*Online Supplementary Figure S1A and B*).

Discussion

This study provides new evidence to show that BCR-ABL1 establishes the MYC/miR-150/MYB/miR-155/PU.1 leukemic pathway in CML pathogenesis. First, we determined the expression levels of these molecules in sorted cell populations from CML-CP patients. Consistent with previous reports[7,8] and our prior data,[10] we observed that miR-150 levels in primary CML samples were lower than those in the corresponding cells of healthy donors.

Next, we investigated the mechanism of miR-150 suppression in CML. Previously, the repression of miR-150 expression by MYC had been demonstrated in an immor-
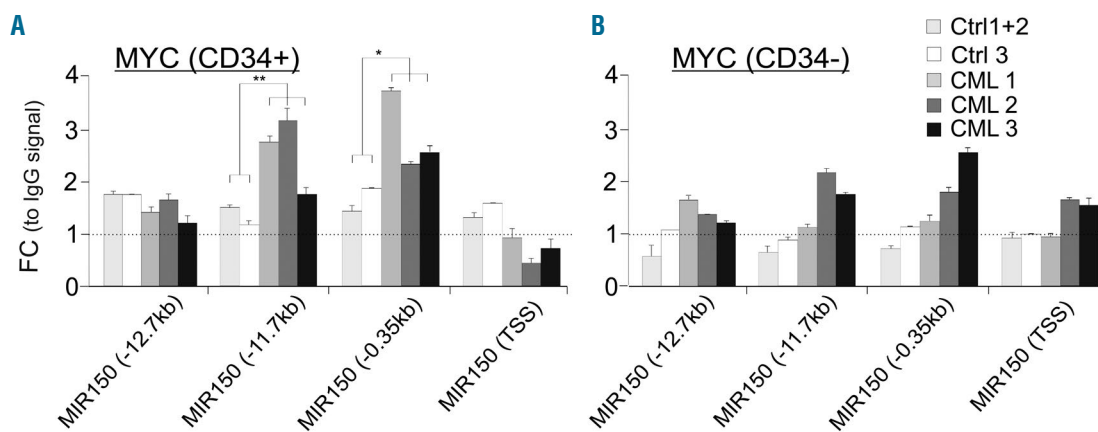

Figure 5. miR-150 regulation by MYC in chronic myeloid leukemia (CML) primary cells. MYC occupancy at putative regulatory loci of the *MIR150* gene in separated (A) CD34+ and (B) CD34- cell subpopulations isolated from healthy donors [control (Ctrl) n=3] and CML-chronic phase (CP) patients at the time of diagnosis (CML; n=3), respectively. Separated cells from 2 healthy donors were pooled due to very low yields. The columns represent the fold change (FC) in the % of DNA input obtained in the leukemic or healthy cells compared with the non-specific IgG precipitation and equalized to 1 (dashed line). Unpaired two-tailed Student *t*-test was used to determine *P*-values. *P<0.05, **P<0.01. Error bars represent standard deviations.

talized human B-cell model; however, the tested mechanism of direct MYC binding to the regulatory loci of the MIR150 gene was not confirmed.[28] In the present study, we identified previously untested loci -11.7 kb and -0.35 kb upstream of the MIR150 TSS that were occupied by MYC in CD34+ and CD34- CML-CP primary cells. The occupancy of MYC at both loci was found in K562 and, in the case of the -11.7 kb locus, in KCL-22 cells but not in BCR-ABL1-negative HL-60 cells. Inhibiting BCR-ABL1 activity with imatinib decreased MYC expression and depleted MYC occupancy at these specific loci in the K562

and KCL-22 cells. In contrast, imatinib treatment had no impact on either *MYC* expression or its occupancy on the studied *MIR150* loci in the HL-60 cells. A pronounced decrease in MYC expression was observed without an impact on BCR-ABL1 activity using JQ1, which significantly increased miR-150 levels in KCL-22 but not K562 cells. This finding may be explained by the assumption that a profound inhibition of both MYC and BCR-ABL1 is required to induce miR-150 expression in K562 cells.

Moreover, we tested dual MYC regulatory mechanisms of *MIR150* expression, including direct transcriptional acti-
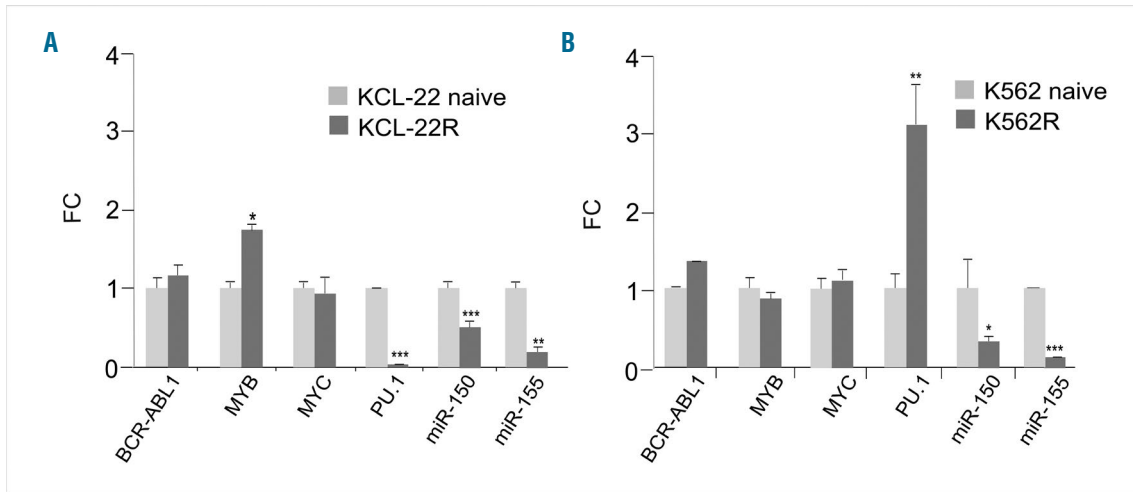
Figure 6. Comparison of the oncogenic pathway expression profiles in imatinib-sensitive (naive) K562, KCL-22 and imatinib-resistant K562R, KCL-22R chronic myeloid leukemia (CML) cell lines. BCR-ABL1, MYB, MYC, PU.1, miR-150 and miR-155 transcript levels in (A) KCL-22 and KCL-22R and in (B) K562 and K562R. Gene expression data for resistant cells represent the fold change (FC; $2^{-\Delta\Delta Ct}$) in relation to corresponding gene expression for naive cells normalized to 1. Unpaired two-tailed Student *t*-test was used to determine *P*-values. *P<0.05, ** P<0.01, and ***P<0.001. Error bars represent standard deviations.
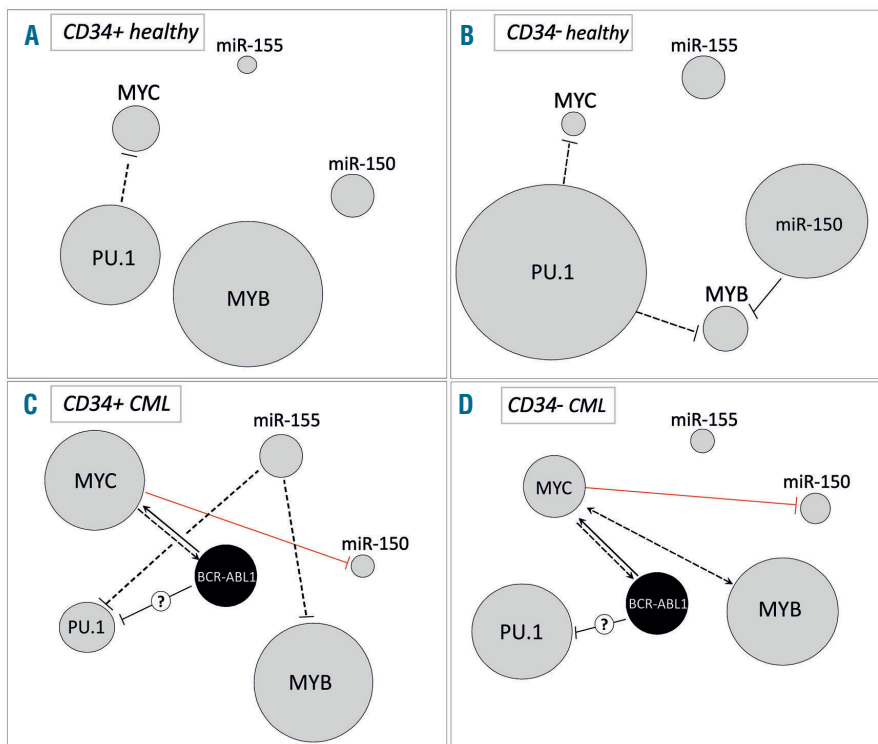
Figure 7. The suggested model of relationships among BCR-ABL1, MYC, MYB, PU.1, miR-150 and miR-155 in CD34+ and CD34- cells from chronic myeloid leukemia (CML) patients and healthy donors. The schemes represent the expression levels and mutual relationships among the molecules in CD34+ (A) and CD34- (B) cells from healthy donors compared with the profiles of CD34+ (C) or CD34- (D) cells, respectively, from CML patients at diagnosis. The circle sizes denote the expression levels of specific molecules. The black lines represent the active stimulatory (arrowheads) or inhibitory (flat-heads) relationships described in the manuscript. The red line emphasizes the novelty mechanism of miR-150 repression by MYC in CML CD34+ cells. By the dashed lines we outlined regulations that we assumed based on published observations.[15,17,18,24,25,27]

vation by MYC and the post-transcriptional inhibition of miR-150 maturation by MYC-driven Lin[28] described using *in vitro* and *ex vivo* AML models with MLL rearrangements.[29] However, our data did not reveal the repression of an miR-150 maturation blockade following the BCR-ABL1 inhibition in CML cells. The specific regulatory mechanisms of miR-150 expression were identified in CML, as we identified MYC-binding loci neighboring the MIR150 TSS in K562 and KCL-22 cells, while there was no MYC binding in AML HL-60 cells. Recently, MYC was reported to exert opposite effects on the expression of a specific gene depending on the local epigenetic pattern;[30] this would be interesting to investigate for the *MIR150* gene given the presence of a CpG island near the -11.7 kb locus.

Together our data suggest that BCR-ABL1 inhibits miR-150 expression in CML cells *via* the transcriptional activation of MYC and its simultaneous recruitment to a specific -11.7 and -0.35 kb loci of the *MIR150* gene, where MYC binds and acts as a direct repressor of miR-150 transcription (Figure 7). This conclusion is reliable since MYC has recently been found to act with p53 as a key CML regulator to maintain CML LSC survival.[31]

Furthermore, our data indicated that low miR-150 levels drive MYB expression in CD34⁻ CML cells and hinder cell differentiation,[9] which is consistent with other reports[32,33] and our finding regarding low PU.1 expression in the primary CML–CP CD34⁺ populations and CD34⁻ CML blast cell lines. Our data showed a significant downregulation of PU.1 following imatinib treatment in K562 cells (a model of erythroid lineage), while the opposite effect on PU.1 expression was observed following imatinib treatment in KCL-22 cells (a model of myeloid lineage), likely reflecting the distinct nature of these cell lines. We hypothesize that BCR-ABL1 inhibition generally relieves blocked cell differentiation by manipulating PU.1 levels because either low or high levels of PU.1 are required for the terminal differentiation of K562 (erythroblasts) and KCL-22 (myeloblasts) cells, respectively. Conversely, both PU.1 upregulation in the K562R imatinib-resistant cells and PU.1 downregulation in the KCL-22R imatinib-resistant cells, together with reduced miR-150 levels, may impose a differentiation block, similar to that described in a murine erythroleukemia (MEL) cell model.[34]

In addition, we found that miR-155 levels in the CD34⁺ CML-CP cells from patients at diagnosis and with TKI resistance were higher than those in healthy CD34⁺ cells. miR-155 was previously identified to be an oncogenic miRNA that is up-regulated in a variety of malignancies.[35-37] Notably, the sustained miR-155 overexpression in the CML was associated with the induction of myeloid disorder in mice.[38] However, the inhibition of BCR-ABL1 activity by imatinib increased miR-155 levels in the KCL-22 and K562 cells, which is consistent with a previous report showing miR-155 upregulation in leukocytes from CML patients following prolonged imatinib treatment.[39] The functionally distinct, dose-dependent effects of miR-155 expression have been recently described in AML, highlighting the importance of the cell context and fine regulation in assessing its role in leukemogenesis.[40]

In summary, we propose a leukemic network model including a novel mechanism of BCR-ABL1-dependent recruitment of MYC oncoprotein to bind and inhibit *MIR150* gene expression in CML cells. Compared with healthy cells, the CD34⁻leukemic cells showed that downregulation of miR-150 expression by MYC resulted in significantly higher MYB levels. miR-150 expression is reduced at the time of TKI resistance and further diminished in resistant CML cell lines, emphasizing the increased aggressiveness of the disease as well as the links between TKI resistance and disrupted cell differentiation. The key connecting nodes of the described leukemic network established by aberrant activity of BCR-ABL1 may serve as potential druggable targets, as was recently shown for the transcription factor MYC[31] to overcome the resistance of CML LSCs.

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