**Cancer Genes and Genomics**

**c-MYC Oncoprotein Dictates Transcriptional Profiles of ATP-Binding Cassette Transporter Genes in Chronic Myelogenous Leukemia CD34+ Hematopoietic Progenitor Cells**

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**Abstract**

Resistance to chemotherapeutic agents remains one of the major impediments to a successful treatment of chronic myeloid leukemia (CML). Misregulation of the activity of a specific group of ATP-binding cassette transporters (ABC) is responsible for reducing the intracellular concentration of drugs in leukemic cells. Moreover, a consistent body of evidence also suggests that ABC transporters play a role in cancer progression beyond the efflux of cytotoxic drugs. Despite a large number of studies that investigated the function of the ABC transporters, little is known about the transcriptional regulation of the ABC genes. Here, we present data showing that the oncoprotein c-MYC is a direct transcriptional regulator of a large set of ABC transporters in CML. Furthermore, molecular analysis carried out in CD34+ hematopoietic cell precursors of 21 CML patients reveals that the overexpression of ABC transporters driven by c-MYC is a peculiar characteristic of the CD34+ population in CML and was not found either in the population of mononuclear cells from which they had been purified nor in CD34+ cells isolated from healthy donors. Finally, we describe how the methylation state of CpG islands may regulate the access of c-MYC to ABCG2 gene promoter, a well-studied gene associated with multidrug resistance in CML, hence, affecting its expression. Taken together, our findings support a model in which c-MYC–driven transcriptional events, combined with epigenetic mechanisms, direct and regulate the expression of ABC genes with possible implications in tumor malignancy and drug efflux in CML. *Mol Cancer Res; 9(8); 1–13. ©2011 AACR.*

**Introduction**

Chronic myeloid leukemia (CML) is probably the most extensively studied human hematopoietic malignancy and accounts for approximately 20% of all leukemias diagnosed in adults (1). In CML, the number of CD34+ progenitor cells represents a useful diagnostic and prognostic indicator regarding the evolution of this disease (2). Furthermore, in CML, CD34+ hematopoietic progenitors carry the t(9;22) (q34;q11) reciprocal chromosomal translocation (chromosome Ph), which gives rise to the BCR-ABL proto-oncogene and its constitutively active protein tyrosine kinase product p210/BCR-ABL (3). Currently, treatment of CML is based on the administration of the tyrosine kinase inhibitors, which selectively inhibit BCR-ABL tyrosine kinase activity responsible for the pathogenesis of this disease (4). However, patients may experience resistance to tyrosine kinase inhibitors, thus limiting the long-term benefits of the drug. The most extensively studied mechanism of drug resistance is represented by point mutations in the BCR-ABL kinase domain that impair tyrosine kinase inhibitors binding (reviewed in ref. 5). Nonetheless, mutations have been found to mediate resistance only in a proportion of resistant patients. Other mechanisms have been invoked but very little is known of their actual role. In this regard, the intracellular concentration of tyrosine kinase inhibitors is a critical feature influencing drug efficacy. Successful therapy of CML is impeded by the development of resistance to a wide spectrum of chemotherapeutic drugs. In a number of cancers, a drug-resistant phenotype has been linked to...
overexpression of some members of the highly conserved family of transmembrane proteins characterized by an ATP-binding cassette (ABC) domain, the so-called ABC superfamily of transporters (6, 7). Some studies have shown that the ABCB1 (Pgp/MDR1) and ABCG2 members of this family are deregulated in imatinib-resistant cell lines and/or patients (8, 9). Less is known on the role of the ABC transporters in mediating resistance to the second-generation tyrosine kinase inhibitors (dasatinib and nilotinib) approved for imatinib-resistant or intolerant CML patients (10–13). Moreover, besides their role in conferring drug resistance, a wide range of observations and correlative studies indicate that high levels of ABC transporters in tumors may determine a malignant progression and a more aggressive phenotype independently of their role in drug efflux (reviewed in ref. 14).

Misregulation of the ABC transporter genes in CML may occur through activity of specific transcription factors whose function is also altered in this disease. MYC dysregulation is considered an oncogenic event required for human tumorigenesis (15–17) and has also been related to the progression of myeloid leukemias (18). The proto-oncogene c-MYC encodes a basic helix-loop-helix leucine zipper transcription factor that, dimerizing with its partner MAX, controls multiple cell functions. Patients with myeloid leukemias are often characterized by the presence of double minute chromosomes that contain MYC amplification (19, 20), and a recent study suggests that several oncogenes, involved in myeloid tumor progression, induce leukemogenesis by activating c-MYC oncoprotein (21). Moreover, in CML, some studies have shown that BCR-ABL can indirectly activate c-MYC function via either the Janus-activated kinase (JAK2) pathway (22) or the mitogen-activated protein kinase/heterogeneous nuclear ribonucleoprotein K (MAPK/HNRPK) pathway (23) causing increased c-MYC mRNA translation. Furthermore, c-MYC is located on chromosome 8 (8q24), and trisomy of chromosome 8 is one of the most frequent additional abnormalities detected in CML patients (24). Overall, these findings point to c-MYC as a main downstream actor in leukemias, and in particular in CML pathogenesis, possibly by modulating transcription of a large set of genes.

Recently, we have established that MYCN is responsible for directing and coordinating the transcription of ABC genes in neuroblastoma tumors (25). Here, we have investigated whether c-MYC can also orchestrate ABC transcription in myeloid leukemias. Importantly, we found that c-MYC is highly expressed in CD34+ progenitor cells from newly diagnosed chronic phase (CP)-CML patients and that its transcriptional level correlates with that of specific ABC transporter genes. Our results show that c-MYC directly controls the transcription of a large subset of ABC transporter genes in myeloid leukemia cells. Finally, we have investigated how, besides c-MYC–mediated transcriptional events, epigenetic mechanisms can lead to dysregulation of ABCG2, a well-studied protein associated with multidrug resistance in CML.

Overall, c-MYC–driven transcription of ABC genes may provide insights into the molecular mechanisms of cancer drug resistance in CML and it may represent an unexpected target for the development of novel therapeutic strategies with important implications for the treatment of different types of leukemia.

Materials and Methods

Cell lines and patient samples

KG-1a cells were cultured in RPMI containing 20% FBS heat inactivated and 50 mg/mL gentamycin. HL-60 and K562 cells were cultured in RPMI medium 1640 containing 10% FBS heat inactivated and 50 mg/mL gentamycin.

Peripheral blood samples from 21 newly diagnosed CP CML patients were collected after written informed consent. Mononuclear cells were obtained by Ficoll–Hypaque density gradient centrifugation. CD34+ cells were selected by using the human CD34 microbead kit on an automatic immunomagnetic separator (AutoMacs, Miltenyi Biotec) according to manufacturer’s recommendations.

Retroviral production and infection of plasmids expressing short hairpin RNAs

To generate short hairpin RNA (shRNA) vectors for c-MYC, hairpin-encoding oligonucleotides were annealed and ligated into pCCL (high titer) expression vector. For c-MYC downregulation, we used the following targeting sequence: 5’-GATGAGGAAGAAATCGATG-3’ previously established in the laboratory of Professor Martin Eilers (26). Retroviruses expressing c-MYC shRNA and nontargeting shRNA were produced in HEK293T cells after cotransfection of pCCL expression vector and pC-φ amphi packaging vector. After 24 hours, supernatants containing the retroviruses are collected and filtered through a 0.45-μm pore size filter. Each supernatant was diluted in 2.5 volume of complete medium. Polybrene (8 μg) per milliliter of medium was added. One day before the infection, the HL-60 cells were seeded such to be at 50% of confluency on the day of infection. On the day of the infection, the medium was replaced with medium containing retrovirus and polybrene. Thirty-six hours after infection, the HL-60 cells were grown in the presence of 8 μg/mL puromycin to select for successful transfectants of the shRNAs. After 10 and 12 days of selection, cells were harvested and analyzed for proteins and RNA expression levels.

Luciferase assay

The pGL3-basic and Renilla-TK vectors were obtained from Promega. Promoters of selected ABC transporter genes were obtained using PCR and cloned into the pGL3-basic vector as described previously (25). The vectors were transfected in HL-60 previously infected with retroviruses expressing c-MYC shRNA and nontargeting shRNA. The transfection was carried out after 10 days of selection with puromycin by using Lipofectamine LTX Reagent according to manufacturer’s recommendations. The activity of firefly or Renilla luciferase was measured after 48 hours with the Dual Luciferase Assay Kit (Promega) according to instructions.
Gene expression analysis

RNA samples were prepared by using Tri-Reagent (Sigma) and treated with DNase (DNA-free, Ambion). Reverse transcription and PCR were carried out by using Thermoscript reverse transcriptase PCR (RT-PCR) kit (Invitrogen). Expression data were obtained both from real-time quantitative PCR (qRT-PCR). qRT-PCR was carried out by using iQ SYBR Green Supermix and the iQ Cycler thermocycler (Bio-Rad). Primers used for qRT-PCR are described in Supplementary Table SII.

Gene expression profile (GEP) was carried out on CD34 + cell fractions obtained from peripheral blood of CML patients at diagnosis by using the Affymetrix HG-U133 Plus 2.0 microarray platform following the manufacturer’s recommendation. Raw data were normalized using the Robust Multichip Average (RMA) algorithm and filtered, and the expression data of ABC transporters and c-MYC were extrapolated from the whole transcriptome. The same approach was applied to the GEP data of normal CD34 + cells, obtained from peripheral blood of healthy donors [Gene Expression Omnibus (GEO) database, accession number GSE12662]. The comparison between the 2 sets of data was carried out by using the \( \Delta \Delta C_t \) method, with the GUSB (housekeeping gene) as internal reference and the medians of each gene expression values as calibrators.

Methylation-sensitive PCR

This protocol was carried out as described previously (35). Genomic DNA was digested with EcoRI or PmlI and then used for PCR. Primers are described in Supplementary Table S2.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) was carried out as previously described (27). Antibodies employed in this study were the following: immunoglobulin G (IgG; Santa Cruz sc-2027), c-MYC (Santa Cruz N-262), and Max (Santa Cruz C-17). Primers for quantitative ChIP are listed in Supplementary Table SII.

Treatment of cells with 5-aza-2’-deoxycytidine

Cells were seeded at a density of 0.5 \( \times 10^6 \) cells/mL and treated with 5 \( \mu \)mol/L of 5-aza-2’-deoxycytidine (AZA) for 72 hours.

Methyl-CpG Immunoprecipitation

The methyl-CpG immunoprecipitation (mCIP) was carried out as previously described (28). The DNA was obtained by using the Blood & Cell DNA culture midi Kit (Qiagen) according to the manufacturer’s instructions. Immunoprecipitation was carried out by incubating 15 \( \mu \)g
of sonicated DNA with 5 μg of 5-methylcytosine monoclonal antibody (Calbiochem NA81).

**Southern blotting**

Genomic DNA (10 μg/lane) was completely digested with EcoRI, EcoRI/MspI, or with EcoRI/HpaII restriction enzymes (New England Biolabs) and loaded onto a 1% agarose gel. DNA was transferred on a positively charged Hybond-XL membrane (Amersham) following a standard alkaline transfer protocol (Current Protocols in Molecular Biology, Wiley). DNA probe was amplified by PCR, gel purified and labeled with α-32P-dCTP by using the Ready-Prime DNA Labeling System (Invitrogen) according to the manufacturer’s instructions. Hybridization and washes were carried out at 65°C using standard procedures.

**Results**

**c-MYC is critical for expression of several ABC transporter genes in promyeloid leukemia cells**

To investigate the potential role played by c-MYC in transcriptional regulation of ABC transporter genes in
myeloid leukemias, we used the promyelocytic leukemia cell line HL-60. Although highly expressing Bcl-2, HL-60 cells were not selected for enhanced resistance to cytotoxic drugs (29). HL-60 cells lack expression of the well-defined human stem cell marker CD34 and they are characterized by c-MYC proto-oncogene amplification. Moreover, c-MYC overexpression can be turned off by treating cells with dimethyl sulfoxide (DMSO; ref. 30). By using this cell model system, we examined the expression level of all 48 human ABC drug transporters as a function of c-MYC silencing at 24- and 48 hours of DMSO treatment. Transcription profiles were determined by qRT-PCR and clustered by using the Cluster 3 program (31). Results show that DMSO-mediated differentiation can affect transcription of a large subset of ABC transporter genes (Fig. 1A). The analysis reveals 4 groups of ABC transporters depending on the correlation of their expression levels with that of c-MYC. For example, a high–c-MYC level correlates with increased expression of a specific group of ABC genes including ABCA2, ABCB9, ABCB10, ABCC1, ABCC4, ABCE1, ABCF1, and ABCF2, a majority of which has been implicated in drug resistance. On the

Figure 2. (Continued).
other hand, the expression of another group of \( ABC \) transporters such as \( ABCA7, ABCB2, ABCD1, ABCD3 \), and \( ABCG1 \) genes inversely correlated with expression of c-MYC. Two additional groups were also identified: one in which genes did not change their expression as a function of differentiation; and a second one in which genes were not at all expressed in HL-60 cells. To further determine whether c-MYC is required to activate or repress transcription of \( ABC \) genes and to rule out pleiotropic effects because of the use of DMSO, we have manipulated c-MYC expression directly and specifically by using a RNA interference approach. HL-60 cells were infected successfully with nontargeting shRNA (shctrl) or c-MYC targeting shRNA (shMYC) retroviruses. The cells were selected in presence of puromycin, and the expression of the subset of \( ABC \) genes previously showed to be responsive to treatment with DMSO was monitored after 10 days and 12 days postselection. Results confirm that c-MYC plays a key role in regulating transcription of a large subset of \( ABC \) transporter genes (Fig. 1B).

**c-MYC is a direct transactivator of a large group of \( ABC \) transporter genes**

Next, we investigated whether c-MYC directly contacts promoters of specific \( ABC \) transporter genes thus affecting their transcription. We mainly focused on those genes that were positively regulated by c-MYC and likely responsible for increased drug efflux. To address this issue, we carried out bioinformatic analysis of the \( ABC \) promoter regions and found out that many of the MYC responsive \( ABC \) gene promoters contain several canonical and noncanonical MYC binding sites (E-box sites), the majority of which are located in regions from −2,000 to +2,000 base pairs around the predicted transcription start site (Fig. 2). To confirm that c-MYC indeed binds these promoters in vivo, we carried out ChIP on HL-60 cells. Results show that promoters of \( ABCA2, ABCB9, ABCB10, ABCC1, ABCC4, ABCE1, ABCF1, \) and \( ABCF2 \) were occupied by the c-MYC/MAX complex (Fig. 2). As a control, ChIP analysis was also employed on HL-60 cells pretreated with DMSO for 3 days, thereby, depleted of the c-MYC protein and found that MAX but not c-MYC was associated with gene promoters (Supplementary Fig. S1). As additional controls, ChIP was carried out for \( ABCA10 \) that does respond to c-MYC, for \( ABCB1 \) and \( ABCG2 \) that are not expressed in HL-60 cells and for \( APEX-1 \), a well-known MYC positive target gene (Fig. 1).

Because direct binding of c-MYC to gene promoters is not per se sufficient to prove its effect on the transcription of target genes (32), we carried out experiments by using luciferase reporter constructs carrying the promoter regions of those \( ABC \) transporters bound by c-MYC in ChIP experiments. Recombinant reporters were transiently cotransfected into HL-60 cells, and luciferase activity was quantified as a function of c-MYC downregulation, obtained through an shRNA strategy and DMSO treatment, respectively (Fig. 3 and Supplementary Fig. S2). As expected, results show that for each of these gene promoters (\( ABCA2, ABCB9, ABCB10, ABCC1, ABCC4, ABCE1, ABCF1, \) and \( ABCF2 \)), luciferase activity was indeed dependent on c-MYC expression. The promoter of \( ABCA10 \) transporter gene was cloned and used as negative control (Fig. 3). Overall, these data support the view that c-MYC may function as a direct transactivator of a large subset of \( ABC \) transporter genes.

**c-MYC controls expression of \( ABC \) transporters in CD34+ hematopoietic progenitors of CML patients**

The expression profiles of c-MYC responsive \( ABC \) transporter genes was analyzed by qRT-PCR in CD34+ progenitors cells versus mononuclear cells of 21 newly diagnosed CP CML patients. Results show that the \( ABC \) genes, whose promoters are bound by c-MYC, were highly expressed in the CD34+ cell population when compared with the population of mononuclear cells from which they

![Figure 3](https://mcr.aacrjournals.org/1541-7786.MCR-10-0510/3.png)

**Figure 3.** The role of c-MYC in \( ABC \) gene transcription is recapitulated by transient transfection assays. \( ABC \) transporter promoters were cloned into a luciferase reporter vector (Luc-reporter). Constructs were tested in HL-60 cells as a function of c-MYC expression (shRNA ctrl and shRNA c-MYC). The \( ABCA10 \) gene was used as a negative control. A renilla reporter cotransfected with each ABC-Luc reporter was used to normalize luciferase activity. Promoter diagram: bent arrow, transcription start site; cloned DNA region (bp) is indicated below promoter map. Results are the mean ± SE of 3 independent transfections.
have been purified, and that their expression strongly correlated with that of c-MYC (Fig. 4A). Furthermore, the GEPs of c-MYC and the ABC transporter genes were extrapolated from the whole transcriptome and compared with those of a CD34⁺ cell population obtained from healthy donors peripheral blood lymphocytes (data obtained from GEO, accession number GSE12662). The median expression values of c-MYC and each ABC transporter gene shows that the CML CD34⁺ cell population expresses significantly higher levels of c-MYC and ABC transporter genes by comparison with healthy donor CD34⁺ cells (Fig. 4B). With regard to ABCG2, we recapitulated the same finding observed for those ABC genes selected as positively regulated by c-MYC. Indeed, we observed higher ABCG2 expression levels in the CML-CD34⁺ cells than in the mononuclear population (Fig. 5A). Interestingly, because ABCG2 promoter is regulated by epigenetic mechanisms, such as DNA methylation (33, 34), by using methylation-sensitive PCR, we found that the ABCG2 promoter was methylated on the MYC

**Figure 4.** c-MYC controls expression of ABC drug transporters in CD34⁺ hematopoietic progenitors. A, relative expression of c-MYC and ABC genes in the CD34⁺ cell population was compared with that of the entire population of mononuclear cells (MNC). Each sample was analyzed in duplicate and threshold cycle (Ct) values were averaged. Results are reported as delta Ct values which were calculated as the difference between Ct of the ABC mRNAs and Ct of the GUSB mRNA used for normalization. B, c-MYC and ABC gene expression of CML CD34⁺ cells was compared with that of CD34⁺ cells from healthy donors. The 2 sets of data were compared by using the ΔΔCt method, with the GUSB (housekeeping gene) as internal reference and the medians of each gene expression values as calibrators. Both in (A) and (B) significant differences between the 2 cell populations were determined by using Mann-Whitney statistical tests.
binding site, located in close proximity to its transcriptional start site, in CD34\(^+\) cells of 5 patients out of the 21 ones that were analyzed (Fig. 5B). Based on this observation, we examined whether there may be a correspondence between the methylation data and the expression level of \(ABCG2\) in our patient samples, and we found that indeed in those patients showing a methylated MYC binding site, the expression of \(ABCG2\) is the lowest (data not shown).

Considering the above, we have re-analyzed microarray data expression of \(ABCG2\) only in those patients that carried an unmethylated \(ABCG2\) gene and compared those data with those of healthy donors (data from GEO database). By doing so, the \(P\) value was definitely improved although it did not reach statistical significance (Fig. 5C, left). Indeed, this trend was then confirmed as statistically significant when \(ABCG2\) expression was measured by qRT-PCR, a more sensitive technique than microchip arrays. In that case, expression of \(ABCG2\) in CML-CD34\(^+\) cells was compared with that of CD34\(^+\) cells from healthy donors extrapolated from GEO database (left) or collected from 10 new healthy donors (right). Significant differences between cell populations were determined by using Mann–Whitney statistical tests.

ChIP was carried out on K562 and KG-1a cell lines, which share several biological and biochemical features with myeloid leukemia cells. K562 derives from a CD34\(^-\)Ph\(^+\)CML, whereas KG-1a derives from a CD34\(^+\)AML. Results show that, in these cells, c-MYC can bind the promoter of all of the \(ABC\) transporter genes found to be positively regulated by c-MYC, fostering the view that c-MYC plays a crucial role in \(ABC\) transporter expression in myeloid leukemias (Fig. 6). In contrast, no binding was observed for \(ABCB1\) or \(ABCA10\), neither of which had been shown to be regulated by c-MYC.

c-MYC–mediated transcription of \(ABCG2\) gene is controlled by its CpG island–promoter methylation status

\(ABCG2\) is usually aberrantly overexpressed on primitive CML CD34\(^+\) HSCs. Indeed, it should be noted that in contrast to what has been found in HL-60 and K562 cells, \(ABCG2\) was expressed both in CD34\(^+\) progenitor cells as well as in KG-1a cells, and its expression correlated with that of c-MYC in CD34\(^+\) progenitors of CML patients (Fig. 5A). Nonetheless, c-MYC was found associated with the \(ABCG2\) promoter in KG-1a CD34\(^+\) cells but not in HL-60 and K562 cell lines (Fig. 6), thus revealing a complex pattern of mechanisms involved in controlling \(ABCG2\) transcription. One possible explanation of these findings may rely on the evidence that the promoter of \(ABCG2\) is often hypermethylated in cancer (33, 34).
Moreover, we have previously shown that MYC cannot bind its cognate sites when methylated (35). Therefore, in the context of promoter methylation, c-MYC does not have access to the \textit{ABCG2} promoter and thus cannot drive \textit{ABCG2} transcription. In support of this hypothesis, it is worth noting that in the HL-60 cell line, c-MYC affects the luciferase activity of a reporter construct carrying the unmethylated promoter of \textit{ABCG2}, but cannot modulate its endogenous expression (Fig. 7A). To better investigate whether a distinct promoter DNA methylation pattern may explain the different \textit{ABCG2} transcriptional levels, KG-1a, HL-60, and K562 cells were treated with AZA, a DNA demethylating agent, and mRNA expression of \textit{ABCG2} was monitored by qRT-PCR. Despite the fact that 3 cell lines show same sensitivity to the treatment with AZA (Supplementary Fig. S3) only in HL-60 and K562 cells was determined by mCIP, by using a monoclonal antibody that specifically recognizes the methylated cytosines. As shown in Fig. 7C, the putative CpG island surrounding the transcription start site of \textit{ABCG2} promoter was found to be enriched in methylated cytosines in HL-60 and K562 cell lines compared with a distal region. As expected, the same region was found to be completely unmethylated in KG-1a cells (Fig. 7C). To confirm these results, the DNA methylation state of the
ABCG2 promoter was evaluated in HL-60 and K562 cells by Southern blot (Supplementary Fig. S4). Genomic DNA extracted from these 2 cell lines was digested with either the MspI or HpaII restriction enzyme and then hybridized with a DNA probe corresponding to the ABCG2 promoter. Although HpaII and MspI recognize the same CCGG sequence, HpaII can cleave that sequence only when unmethylated. As shown in Supplementary Figure S4, the hybridization profile reveals that the ABCG2 promoter region is resistant to cleavage with HpaII but not to the cleavage with MspI, indicating that the ABCG2 promoter region is highly methylated in HL-60 and K562 cell lines.

Overall, the combined ChIP data along with the methylation data allow us to conclude that c-MYC can target ABCG2 expression only when its cognate sites located within ABCG2 promoter are unmethylated and importantly, in the context of different types of leukemia, this event seems to occur prevalently, if not exclusively, in CD34⁺ progenitor hematopoietic cells as shown in Fig. 5B.

Discussion

Resistance to chemotherapeutic agents is a major obstacle for the successful treatment of cancer. The failure of the curative treatment of cancer patients often occurs as a result of intrinsic or acquired drug resistance of tumors to chemotherapeutic agents. Therefore, understanding how chemoresistance develops and eventually how it can be prevented, becomes crucial to fighting cancer effectively. Functional redundancy in the ABC transporters family complicates their utility as therapeutical targets. In this study, we have provided lines of evidence that c-MYC can target a wide spectrum of ABC transporter genes with different structural and functional features raising questions as to how their altered expression may be relevant for drug efflux and more in general for tumor biology. In particular, our data suggest that c-MYC may contribute to the multidrug resistance profile and malignant progression of myeloid tumors by dysregulating the transcription of specific ABC transporter genes prevalently in CD34⁺ hematopoietic cells.
progenitors of CML patients. The fact that BCR-ABL has been previously shown to increase c-MYC expression (22, 23) and that c-MYC in turn coordinates transcription of a large set of ABC transporter genes, reveals a novel role of BCR-ABL in potentially mediating drug efflux in CML. Numerous studies have shown that members of the ABC family shown here to be positively regulated by c-MYC may be involved in the development of a chemoresistance phenotype. ABCC1, ABCC4, and ABCG2 can confer resistance to several antineoplastic drugs such as antifolates, doxorubicin, and nucleotide analogs (36). In addition, the ABCA2 transporter has been associated with drug resistance in childhood T-cell acute lymphoblastic leukemia (37), whereas ABCE1 gene, an inhibitor of Ribonuclease L, was amplified in several tumorigenic cell lines and its high expression correlated with resistance to macrolide inhibitors of protein synthesis (38). Furthermore, ABC transporters play specific roles in cancer biology beyond the efflux of cytotoxic drugs, contributing to some of cancer hallmarks, such as evasion of apoptosis, limitless replicative potential, tissue invasion, and metastasis (reviewed in ref. 14). Indeed, ABCC4 is involved in human dendritic cell migration (39) and its expression is affected during hematopoietic stem cell differentiation (40), whereas ABCF2 has been found amplified in ovary and breast cancers suggesting a possible role in malignant progression (41). Likewise, ABCG2 is highly expressed in a wide range of stem cells (reviewed in ref. 42) and in primitive hematopoietic CML CD34^+ progenitors (9). ABCG2 is responsible to establish the side population phenotype of HSCs (43), blocking hematopoietic development, and maintaining the stem cell self-renewal potential. It has been shown that BCR-ABL TK inhibitors exhibit high affinity interaction with ABCG2 in HSCs (44, 9, 12). Although a considerable debate is ongoing about the role played by ABCG2 in mediating imatinib resistance, a recent study shows that ABCG2-transduced K562 cells are protected from imatinib-induced cell death, suggesting that ABCG2 overexpression can indeed trigger resistance to tyrosine kinase inhibitors in CML stem cells (12). In support of this evidence, it has also been shown that overexpression of

Figure 7. Methylation status of the ABCG2 promoter. A, ABCG2 transporter promoters were cloned into a luciferase reporter vector and its activity was tested in HL-60 cells as function of c-MYC downregulation. B transcriptional reactivation of ABCG2 following the treatment of HL-60 and K562 cells with 5 μmol/L AZA for 72 hours. ABCG2 mRNA expression was determined by qRT-PCR and compared between control (–AZA) and treatment (+AZA). Results are expressed as fold difference between control and +AZA condition and setting to 1 the expression of ABCG2 in the control condition. C, mCIP was employed in KG-1a, HL-60, and K562 cell lines to determine the methylation status of the ABCG2 promoter in vivo. Position of the putative CpG island is shown schematically with the ABCG2 gene diagram (left). A and B represent a region distal from the transcription start site and the putative CpG island surrounding the transcription start site (TSS), respectively. Relative enrichment obtained with the specific antibody was compared with that obtained with preserum (IgG) which was set to 1 in the graph. Results are the average of 4 independent dual ChIP experiments.
ABCG2 in HEK293 cells results in a reduced intracellular imatinib accumulation (45) and in addition, in vitro studies have confirmed that imatinib is a substrate for transport by ABCG2 (46, 47). It has been previously reported that in human renal carcinoma as well as in multiple myeloma cell lines, alterations in the methylation state of the chromatin-embedded ABCG2 promoter have a dramatic effect on its expression profile (33, 34). Here, we provide a rationale of how epigenetic control of c-MYC–mediated transcription can lead to ABCG2 upregulation in CD34+ CML stem cells but not in committed progenitor and mature blood cells. Thus, a combination of transcriptional and epigenetic mechanisms may explain the altered expression of ABCG2, and more in general of ABC transporter genes, hence conferring peculiar and specific drug resistance profiles in different types of leukemia.

Particularly interesting is the finding that a fraction of CML patients displays methylation of the ABCG2 promoter in CD34+ progenitor cells, suggesting that these patients may better respond to chemotherapeutic drugs.

Overall, our findings suggest that deregulation of a specific set of ABC transporters may play a crucial role in dictating a drug resistance phenotype and tumor malignancy in CML with numerous implications for CML treatment. Firstly, increased expression of ABC drugs transporters by the BCR-ABL/c-MYC network, particularly in the cancer stem cell population, may explain for their self-renewal potential and for less sensitivity to drug treatments. This last observation is consistent with a recent view on how drug resistance can be mediated by stem cells. According to this model, tumors contain a small population of cancer stem cells that produce differentiated offspring that are committed to a particular lineage. Following chemotherapy, the committed cells are killed, but the stem cells, which express a variety of drug resistance mechanisms, survive and can repopulate the tumor (48). Therefore, understanding the molecular bases for drug resistance of HSCs may help devise novel therapeutic interventions aimed at a complete eradication of minimal residual disease. Secondly, upregulation of the ABC genes in the HSC population may predispose cancer cells to developing drug resistance following chemotherapy treatment, particularly when a low dosage of the drug is employed, which may facilitate positive selection and expansion of cells with high drug efflux capacity.

In conclusion, our study unveils novel aspects of the BCR-ABL/c-MYC cross-talk that may have important implications for drug resistance and risk assessment in CML.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank D. Manzoni for excellent work with cell cultures.

**Grant Support**

This work was supported by The Italian Association for Research on Cancer (ABR, Italy), The Italian Ministry of University and Research (Italy) and University of Bologna (Italy), The National Health and Medical Research Council, Australia (M.D. Norris, M. Haber) and the Cancer Institute New South Wales, Australia (M.D. Norris, M. Haber) Children's Cancer Institute Australia for Medical Research is affiliated with the University of New South Wales and Sydney Children's Hospital.

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Received November 11, 2010; revised June 9, 2011; accepted June 14, 2011; published OnlineFirst June 21, 2011.

**References**


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Mol Cancer Res  Published OnlineFirst June 21, 2011.