ARTICLE
Journal of Cellular Biochemistry 116:589–597 (2015)

Journal of Cellular Biochemistry

DNA Methyltransferase 1 Drives Transcriptional Down-Modulation of β Catenin Antagonist Chibby1 Associated With the *BCR-ABL1* Gene of Chronic Myeloid Leukemia

Elisa Leo,* Manuela Mancini, Fausto Castagnetti, Gabriele Gugliotta, Maria Alessandra Santucci, and Giovanni Martinelli

Dipartimento di Medicina Specialistica, Diagnostica a Sperimentale-DIMES, Istituto di Ematologia "L. e A. Seràgnoli" – University of Bologna – Medical School, Bologna, Italy

ABSTRACT

The decrease of Chibby1 (CBY1) contributes to β catenin constitutive activation associated with the presence of the *BCR-ABL1* fusion gene of chronic myeloid leukemia (CML). This is mediated by transcriptional events and driven by DNA hyper-methylation at promoter-associated CpG islands of the CBY1-encoding gene *C22orf2*. Moreover, CBY1 transcriptional induction proceeding from promoter de-methylation is a component of *BCR-ABL1*+ cell response to Imatinib (IM). Our study showed that DNA methyltransferase 1 (DNMT1) has a central role in the hyper-methylation at the *C22orf2* promoter. Further investigation in leukemic hematopoietic progenitors from IM-responsive and IM-resistant CML patients at diagnosis failed to demonstrate any correlation between DNMT1-driven hyper-methylation of the *C22orf2* promoter and response to IM. Notably, the response to IM was neither predicted by DNMT1-driven hyper-methylation of *BCL2-like11* at diagnosis. In conclusion, the hypermethylation of *C22orf2* and *BCL2-like11 promoters* proceeding from DNMT1 is a crucial component of their reduced expression, but it is not directly involved in CML resistance to IM. It might rather contribute to the disease evolution towards a drug-resistant phenotype in more advanced phases or blast crisis. J. Cell. Biochem. 116: 589–597, 2015.

KEY WORDS: CHRONIC MYELOID LEUKEMIA; *BCR-ABL1*; CHIBBY1; DNA METHYLTRANSFERASE 1; BCL2-LIKE11

The conversion of inactive *ABL* into the constitutively active *ABL* isoform of *BCR-ABL1* fusion protein is the causative event of chronic myeloid leukemia (CML). It is driven by the tetramer domain at the *BCR* N-terminus [McWhirter et al., 1993]. The majority of CML patients achieve a complete cytogenetic remission in response to TK inhibitor IM [Druker et al., 2006], but up to one third of CML patients develop primary or secondary drug resistance [Roychowdhury and Talpaz, 2011]. In most instances, CML resistance to Imatinib.

(IM) and second generation TK inhibitors is caused by *BCR-ABL1*-dependent mechanisms, including gene amplification and mutations. *BCR-ABL1*-independent mechanisms, involving alternative pathways downstream of *BCR-ABL1*, may drive a clonal evolution of the leukemic clone towards drug resistance [La Roseée and Hochhaus, 2008]. Notably, the leukemic stem cell (LSC) compartment, whose self-renewal potential has a central role in sustaining the expansion of clonal hematopoiesis, does not depend from *BCR-ABL1* TK for proliferation and survival and is resistant in vitro to IM and the second generation TK inhibitors nilotinib and dasatinib [Graham et al., 2002; Copland et al., 2006; Jorgensen et al., 2007; Lemoli et al., 2009; Corbin et al., 2011; Hamilton et al., 2012]. This unique trait is now regarded as one major cause of disease persistence under therapy and drug resistance [Chu et al., 2011; Iqbal et al., 2013].

β Catenin is a crucial signal for self-renewal and persistence under TK inhibitor therapy of *BCR-ABL1* + LSC [Zhao et al., 2007; Hu et al., 2009]. Moreover, it has a role in clonal evolution of CML from chronic phase to blast crisis [Jamieson et al., 2004; Minami et al., 2008]. Multiple events concur to β catenin stabilization and activation associated with *BCR-ABL1*. They encompass post-transcriptional modifications resulting in β catenin impaired degradation and/or deregulated transcription of genes whose proteins are involved in β catenin activation [Coluccia et al., 2007; Huang et al., 2010; Huang et al., 2013]. β Catenin increase and

Conflicts of interest: The authors declare that they have no competing interests.
Grant sponsor: Umberto Veronesi Foundation; Grant sponsor: FP7, NGS-PTL.
*Correspondence to: Elisa Leo, PhD, DIMES, Istituto di Ematologia "L. e A. Seràgnoli" – University of Bologna – Medical School, Via Massarenti 9, 40138 Bologna, Italy. E-mail: elisa.leo1@gmail.com
Manuscript Received: 30 May 2014; Manuscript Accepted: 4 November 2014
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 11 November 2014
DOI 10.1002/jcb.25010 • © 2014 Wiley Periodicals, Inc.

its nuclear translocation result in the binding of β catenin with the T-cell factor/lymphoid enhancer factor 1 (TCF/LEF1) transcription factors to form a transcriptionally active complex which triggers critical target genes for leukemic progenitor proliferative advantage [Henderson and Fagotto, 2002; Holland et al., 2013]. Our recent study established that a further component of β catenin activation is its antagonist CBY1 [Leo et al., 2013].

CBY1 is a small protein which interacts with the β catenin C-terminal activation domain hence hampering its binding with TCF/LEF1 transcription factors and drives β catenin nuclear export in a tripartite complex encompassing 14-3-3 scaffolding proteins [Takemaru et al., 2003; Li et al., 2008]. Its down-modulation in the putative LSC compartment of CML identified by a CD34+ phenotype is associated with β catenin nuclear localization and transcriptional activity [Leo et al., 2013]. *BCR-ABL1*-associated down-modulation of CBY1 is evoked by transcriptional events and driven by DNA hyper-methylation at promoter-associated CpG islands of the CBY1-encoding gene *C22orf2* [Leo et al., 2013]. Notably, β catenin inactivation in response to IM in*BCR-ABL1*+ cells is at least partly driven by CBY1 induction proceeding from *C22orf2* promoter de-methylation[Mancini et al., 2013].

DNA methylation is a key epigenetic modification for cellular processes, such as X-chromosome inactivation, imprinting, and transcriptional silencing of specific genes and repetitive elements. In mammals, it predominantly occurs at the CpG dinucleotides, where DNMTs mediate the transfer of methyl groups to cytosines hence generating 5-methylcytosines (5mC). High density CpG dinucleotides, cover transcription initiation sites of approximately 70% of annotated gene promoters. Their methylation correlates with transcriptional silencing mediated by interference(s) with transcription factor binding. The CpG island methylator phenotype encompassing genes involved in leukemic cell proliferation and survival is a common event in CML, eventually associated with the disease progression and drug resistance outcome [Janssen et al., 2010; Jelinek et al., 2011; Leo and Martinelli, 2014].

The aim of our study was to investigate the role of DNMT1 in *BCR-ABL1*-associated hyper-methylation of *C22orf2* and the *BCL2-like11* promoters and CML response to TK inhibitors.

MATERIALS AND METHODS

CELLS AND TREATMENTS

The *BCR-ABL1*+ cell line K562, originated from a CML patient in blast crisis, was used for our preliminary experiments. Biomolecular analyses in K562 cells treated with IM (2 μM) and the de-methylating agent 5-aza-2′-deoxycytidine (5-Aza-CdR) (0.5 μM) were carried to correlate the impact of DNMT1 recruitment at the *C22orf2* promoter with its hyper-methylation and transcription rate. To investigate the putative correlation between *C22orf2* promoter hyper-methylation and transcription rate with the therapy outcomes, five CML patients in major molecular response (MMR) to IM and three CML patients who developed IM resistance while on treatment were analyzed. All the patients were in chronic phase. Mononuclear cell fractions (MCFs) were isolated from bone marrow samples by Ficoll-Hypaque (GE Healthcare) technique. Cells were preliminarily assayed for *BCR-ABL1* mutations by direct sequencing (data not shown). Pooled MCFs from peripheral blood samples of eight healthy persons (HPs) were used as controls for transcript and protein levels and epigenetic chromatin modifications. Preliminary experiments let us to exclude any significant difference in gene expression and epigenetics relative to the cell source (data not shown). Clinical samples and data were collected according to the guidelines of clinical trials NCT00769327, NCT01535391, and NCT01061177 (ref. clinicaltrials.gov) approved by the Ethical Committee of the Policlinico S. Orsola-Malpighi on July 15, 2008, September 13, 2011, and June 29, 2010, respectively. Participants were informed of the general purpose of our study and that the data obtained from their samples would be kept anonymous and unlinked from clinical databases.

RNA ANALYSIS

Total RNA was extracted using a commercial kit (SV total RNA Isolation System from Promega) according to the manufacturer's instructions. RT reaction was carried on 1 μg of total RNA using ImProm-II Reverse Transcription System (Promega). The RT reaction was performed in a final volume of 50 μl containing 1 μg of total extracted RNA, 10 μl of ImProm-II 5X Reaction Buffer, 3 mM of $MgCl_2$, 0.5 mM of each dNTPs, 20 U of Recombinant RNasin Ribonuclease Inhibitor, and 0.5 μg of random hexamers. The RT reaction contemplates an initial step at 25°C for 5′ followed by 40°C for 80′ and 72°C for 15′.

PCR amplifications were performed with 1.25 U of Taq DNA Polymerase kit (Roche) in 30 μl of reaction buffer containing 0.4 μM of each primer, 0.2 mM dNTPs and 5 μl of RT product. PCR was performed after a 4′ denaturation step at 95°C, followed by 32 cycles with a denaturation step at 95°C for 30″, a primer annealing step at 59°C (CBY1 and beta-2-microglobulin [B2M]) and 60°C [*BCL2-like11]* for 30″ and an elongation step at 72°C for 30″. The following primers were used: 5′-AGAGTCCTTGCTGGGGGTTCG-3′ (forward) and5′-CTCCACCTCCCGGGTTGATCG-3′ (reverse) to amplify the two isoforms (200 and 340 bp) of CBY1, 5′-GCCAAGCAACCTTCTGATG-3′ (forward) and 5′-AATACCCACTG-GAGGACCG-3′ (reverse) to amplify a 286 bp region of *BCL2-like11* and5′-CTCGCGCTACTCTCTCTTTCT-3′ (forward) and 5′-TCA-CATGGTTCACACGGCAGGC-3′ (reverse) to amplify a 289 bp region of B2M, as control for RT efficiency.

PROTEIN ANALYSIS

Western blot (WB) analyses were performed on whole cell lysates according to published methods [Mancini et al., 2010]. The anti-ABL antibody recognizing the phosphorylated isoform at tyrosine residue (Y^{245}) within the SH2-domain, proceeding from Y^{412} phosphorylation in the activation loop responsible for BCR-ABL1 constitutive TK activity, was purchased by Cell Signaling Technology. The anti-CBY1 antibody was kindly provided by Ken-Ichi Takemaru [Takemaru et al., 2003]. The anti-β-catenin and -cyclin D1 antibodies were purchased from Cell Signaling Technology. The anti-BCL2-like11 antibody was purchased from Oncogene. The anti-beta actin antibody used as a control for protein loading was purchased from Santa Cruz Biotechnology. Signal intensities in single blots obtained in three separate experiments were measured

by means of ChemiDoc-It instrument equipped with dedicated software (Launch VisionWorksLS, Euroclone). The differences among signal intensities were evaluated for statistical significance using the paired Student's *t*-test.

CHROMATIN IMMUNOPRECIPITATION (ChIP)

Cells were fixed in RPMI at 1% final concentration of formaldehyde. After 10 min incubation at room temperature the reaction was stopped by the addition of 1.25 mM glycine. ChIP was performed using a commercial kit (EpiQuik Chromatin Immunoprecipitation Kit from Epigentek) using an anti-5 methylcytosine (5mC) from ZymoResearch and an anti-DNMT1 (Sigma) ChIP grade antibodies. Fifty ng eluted DNA were then amplified by PCR (initial denaturation 95°C for 10′, 35 cycles: denaturation: 95°C for 30″, annealing 58°C for 30″ and elongation at 72°C for 30″) using FastStart Taq Polymerase Kit (Roche). The following primers were used F-5′ AGGTCAGTGATCCAGCTGCTTGT 3′ and R-5′ ACTCATGCTG-CACACCCGGC 3′to amplify a 205 bp promoter region encompassing nucleotides −85 to +120 of *C22orf2* promoter. PCR amplification of a 342 bp sequence of *BCL2-like11* promoter (at an ATG internal region of 358 bp encompassing nucleotides −268 to +90 critical for gene transcription) was performed on DNA from ChIP products using the following primers:5′ ACTCCACAAGCTGGGGAGCTGAT 3′ (forward) and 5′AGGCCTCTCAGCAGGCTGCAATT 3′ (reverse).

RESULTS

The aim of the study presented here was to investigate the role of DNMT1 in *BCR-ABL1*-associated hyper-methylation of *C22orf2* promoter and CML response to TK inhibitors. Preliminary experiments carried in the Philadelphia 1 (Ph1)+ cell line K562 showed that CBY1 transcriptional induction following *BCR-ABL1* TK inhibition by IM is associated with *C22orf2* promoter de-methylation and persistent reduction of DNMT1 at a 205 bp sequence of *C22orf2* promoter encompassing nucleotides −85 to +120 ($P < 0.05$ or less) (Fig. 1A). Both 5mC and DNMT1 reduction at the *C22orf2* promoter were early events, already apparent at first hour of drug exposure, and persisting up 24th hour, when CBY1 transcript was already declining (Fig. 1A). By the time, CBY1 protein expression was still significantly higher compared to the untreated control ($P < 0.05$ or less), supporting the contribution of post-transcriptional events affecting the protein stability to CBY1 down-regulation by *BCR-ABL1* TK [Leo et al., 2013]. The participation of DNMT1 release from *C22orf2* promoter in CBY1 transcriptional induction was supported by results obtained with the de-methylating agent 5-Aza-CdR, a drug promoting selective DNMT1 degradation, but not affecting *BCR-ABL1* TK [Ghoshal et al., 2005; Mancini et al., 2013]. As expected, 5-Aza-CdR (0.5 μM) induced a persistent and significant reduction of DNMT1 recruitment at the above mentioned *C22orf2* promoter region since 24th up to 72nd hour, associated with 5mC decrease and CBY1 transcript increment ($P < 0.05$ or less) (Fig. 1B). Notably, C22*orf*2 promoter de-methylation in response to 5-Aza-CdR required a longer interval (24 h) compared to IM (1 h) (Fig. 1B). The findings strengthen the central role of *BCR-ABL1* TK activity in the epigenetic control of gene expression. Notably, C22*orf*2 promoter de-methylation persisted up to 24th hour of IM treatment and 48th hour of 5-Aza-CdR treatment, when CBY1 transcript already declined (Fig. 1A,B). The findings suggest that *BCR-ABL1*-associated chromatin methylation status is controlled by further signals in addition to DNMT1.

CBY1 epigenetic down-regulation may play a role in CML prognosis, eventually proceeding from β catenin increase and activation. The putative impact of CBY1 expression on CML responsiveness to IM was investigated in MCFs from bone marrow samples of five CML patients who exhibited a MMR to IM and three patients who developed IM resistance (not contingent upon *BCR-ABL1* point mutations) during treatment. In both groups of patients CBY1 expression levels, 5mC content and DNMT1 recruitment at the *C22orf2* promoter were assessed at clinical diagnosis. Clinical characteristics of patients included in the study are illustrated in Table I. MCFs from peripheral blood apheresis from eight healthy persons (HPs pooled to avoid individual differences) were used as control for gene expression and chromatin epigenetics. Three out of five IM-responsive patients exhibited a significant reduction of both CBY1 transcript isoforms compared to normal control pool ($P < 0.05$ or less) (Fig. 2A). CBY1 transcriptional down-modulation was associated with significant enhancement of DNMT1 recruitment and 5mC content at the *C22orf2* promoter compared to the HP pool ($P < 0.005$ or less) (Fig. 2A). It matched significantly lower expression of the CBY1 protein ($P < 0.001$ or less) (Fig. 2B). The findings support the contribution of post-transcriptional events affecting the protein stability to CBY1 reduction associated with *BCR-ABL1* [Leo et al., 2013]. As expected, CBY1 reduced expression was related with higher levels of β catenin and cyclin D1, a direct target of the β catenin/TCF complex critical for deregulated proliferation of *BCR-ABL1*-transformed cells ($P < 0.05$ or less) (Fig. 2B) [Afar et al., 1995]. All three CML patients who developed IM resistance during treatment exhibited a similar reduction of CBY1 transcript compared to HP pool ($P < 0.05$ or less) (Fig. 3A). As in IM-sensitive patients, CBY1 transcriptional down-modulation was associated with enhanced DNMT1 recruitment at the *C22orf2* promoter and hyper-methylation ($P < 0.01$ or less) (Fig. 3A). Moreover, it paralleled a much greater reduction of CBY1 protein and a significant increase of β catenin and cyclin D1 ($P < 0.001$ or less) (Fig. 3B). Our results confirmed that *C22orf2* promoter hyper-methylation is a central component of CBY1 transcriptional down-modulation in more differentiated hematopoietic progenitors of CML as in the putative LSC compartment identified by the CD34+ phenotype and let advance DNMT1 participation in such epigenetic modification [Leo et al., 2013]. However, neither CBY1 down-modulation nor DNMT1-driven *C22orf2* promoter hyper-methylation at the moment of clinical diagnosis are predictive of CML prognosis and, in particular, of the disease responsiveness to IM.

BCL2-like11 was chosen for further investigation on the role of *BCR-ABL1*-associated methylator phenotype at tumor suppressor genes in CML responsiveness to IM. Here we show that both gene transcript and product were significantly reduced in MCFs from bone marrow samples of IM-resistant patients at the moment of clinical diagnosis compared to normal control ($P < 0.0001$ or less) (Fig. 4). As

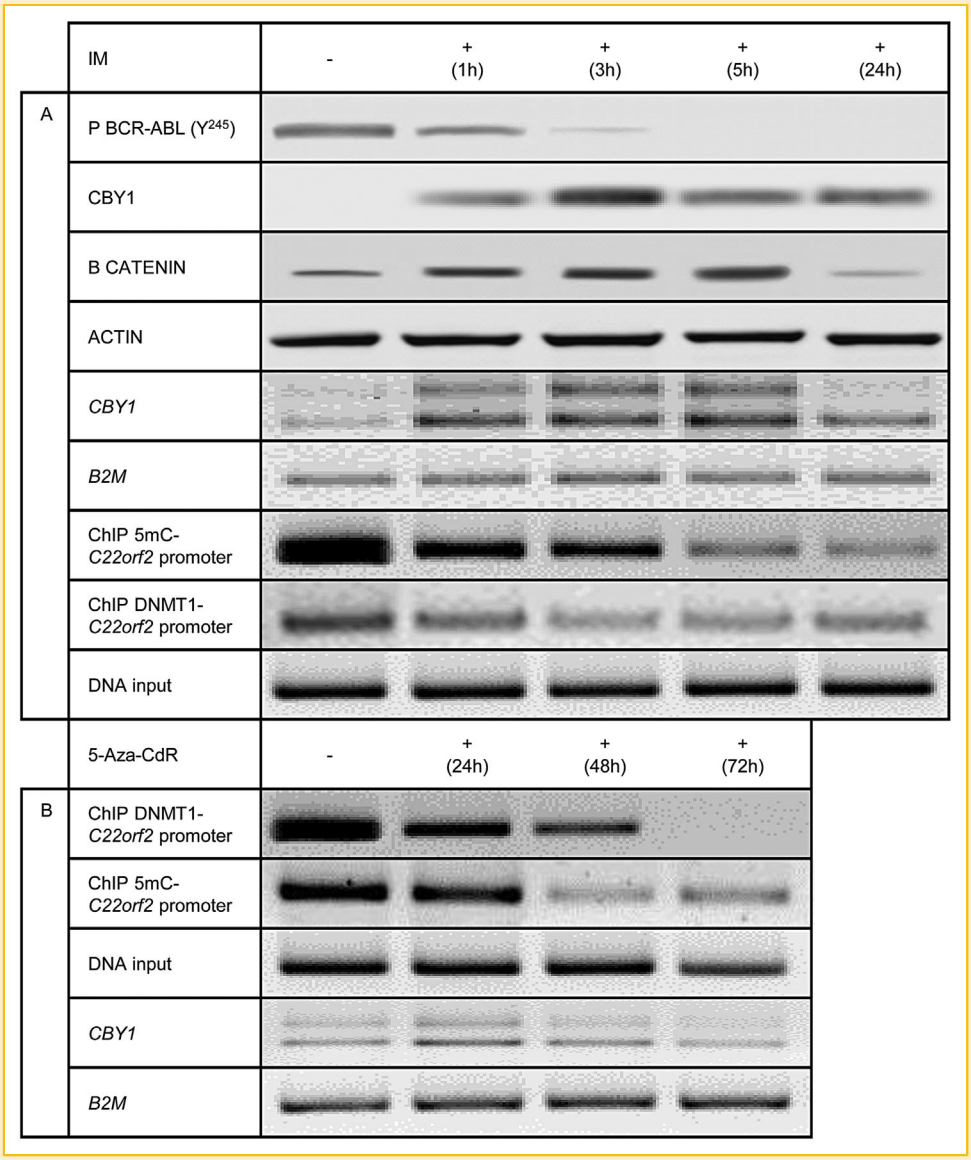

Fig. 1. DNMT1-driven hyper-methylation of the *C22orf2* promoter is a component of CBY1 transcriptional down-modulation associated with BCR-ABL1 TK. (A) In BCR-ABL1+ cell line K562 (which exhibits low levels of CBY1 transcript and no protein) p210 BCR-ABL1 inhibition in response to IM was associated with a significant increment of CBY1 protein and transcript ($P < 0.05$ or less) and β catenin protein up to 5th h ($P < 0.01$ or less) of IM treatment. β Catenin expression underwent a significant reduction ($P < 0.01$) likely contingent upon degradation by the APC/Axin/GSK3β destruction complex. CBY1 transcriptional increment is contingent upon a significant reduction of 5 mC content and DNMT1 recruitment at 205 bp region encompassing nucleotides -85 to +120 of *C22orf2* promoter since 1st up to 24th h of treatment with 2 μM IM ($P < 0.05$ or less); (B) DNMT1 release from the above mentioned region of *C22orf2* promoter resulted in its de-methylation and CBY1 transcriptional induction ($P < 0.05$ or less) following treatment with 0.5 μM 5-Aza-CdR, a drug promoting selective DNMT1 degradation but not affecting the BCR-ABL1 TK. Results presented here have been confirmed in three individual experiments. Signal intensities and statistical significance of differences have been measured as described in detail in the Materials and Methods section. DNA input was used as control of DNA amplification after chromatin Immunoprecipitation (ChIP) assay. B2M served as control for retro-transcription and PCR.

in the case of IM-sensitive patients *BCL2-like*11 transcriptional down-modulation was associated with the hyper-methylation of *BCL2-like11* promoter at a 342 bp sequence encompassing nucleotides −268 to +90 and contingent upon significantly enhanced recruitment of DNMT1 ($P < 0.001$ or less) (Fig. 4). Those results let conclude that DNMT1 has a central role in *C22orf2* and *BCL2-like11* promoter hyper-methylation, resulting in gene transcriptional down-modulation. However, DNMT1-driven hyper-methylation of both gene promoters at diagnosis is not predictive of IM-resistance outcome.

DISCUSSION

CML is caused by a single genetic lesion, the *BCR-ABL1* fusion gene, and may be cured with drugs targeting the constitutive TK activity of

TABLE I. Characteristics of IM-Responsive (S1 Through S5) and IM-Resistant (R1 Through R3) CML Patients Included in our Study

Patient	Sokal score	CR	MMR	2nd line therapy	Best CR	MMR
S1	Low	CCR	Yes	/	/	/
S2	Low	CCR	Yes	/	/	/
S3	Low	CCR	Yes	/	/	/
S4	Inter	CCR	Yes	/	/	/
S5	High	CCR	Yes	/	/	/
R1	High	No	No	Dasatinib	No	No
R2	High	No	No	Nilotinib	No	No
R3	High	Minimal	No	Dasatinib	Minimal	No

The disease prognosis was expressed by Sokal score as low, intermediate or high risk of progression [Sokal et al., 1988]. The cytogenetic response (CR) to IM treatment was complete (CCR) in five IM-responsive patients, absent or minimal in three IM-resistant patients. Accordingly, major molecular response (MMR) was only seen in the former group of patients. IM-resistant patients were enrolled in 2nd line therapy protocols with either dasatinib or nilotinib, but did not achieve any CR or MMR.

BCR-ABL1 fusion proteins [McWhirter et al., 1993; Druker et al., 2006] Additional signals, supporting proliferation and giving a survival advantage to the clonal hematopoiesis, may be implicated in the disease progression and in the resistance to TK inhibitors [La Roseée and Hochhaus, 2008]. In particular, β catenin has been involved in the long-term renewal of LSC and reprogramming of more mature granulocyte-macrophagic progenitors towards stemness [Jamieson et al., 2004; Zhao et al., 2007; Minami et al., 2008; Hu et al., 2009]. In such cell context, β catenin may therefore act as a putative signal of resistance to TK inhibitors and be central in the disease relapse. Events contributing to β catenin activation in CML involve β catenin stabilization by *BCR-ABL1* phosphorylation at tyrosine residues 86 and 654 (which impairs the recruitment by adenomatous polyposis coli [APC]/Axin/glycogen synthase kinase 3 β [GSK3 β] destruction complex), overexpression of growth arrest specific 2 (GAS2, which reduces the calpain-dependent degradation), and GSK3β inactivation due to the prevalence of a GSK3β mis-spliced isoform unable to phosphorylate β catenin and/or to GSK3β de-phosphorylation by the Fas-associated phosphatase 1 (Fap1) [Coluccia et al., 2007; Huang et al., 2010, 2013]. CBY1, a β catenin

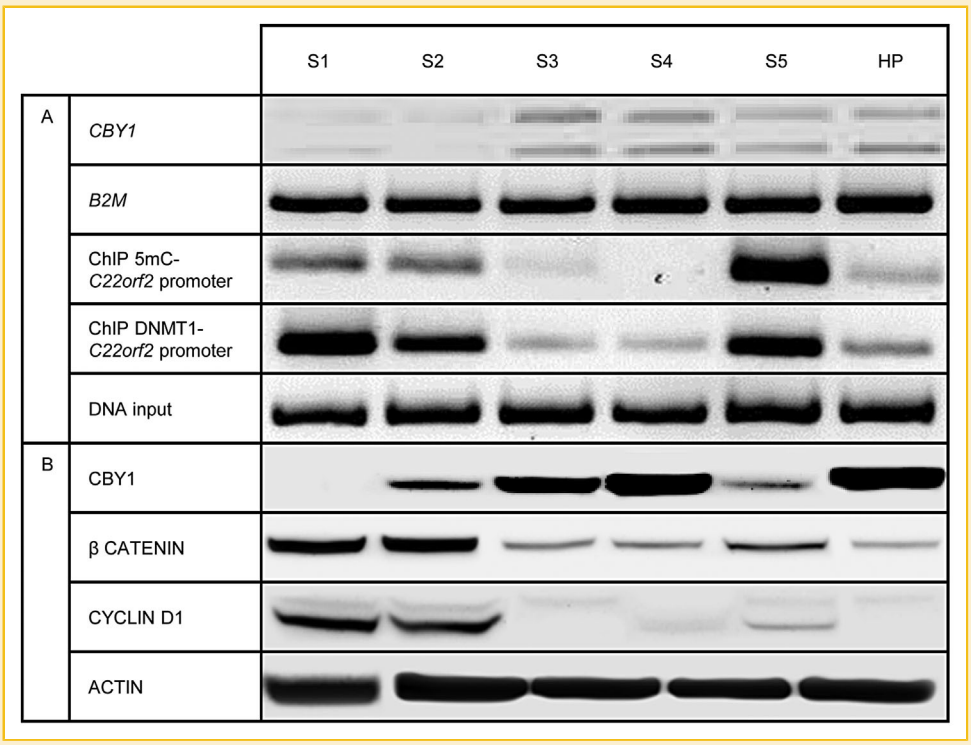

Fig. 2. DNMT1 recruitment leading to hyper-methylation of *C22orf2* promoter and CBY1 transcript reduction at the moment of diagnosis in CML patients who responded to IM therapy. (A) At diagnosis, CBY1 transcript reduction compared with normal control (HP, pooled RNA from peripheral blood MCFs of eight HPs) was apparent in three out of five CML patients who responded to IM therapy (S1, S2 and S5) ($P < 0.05$ or less). In all cases it was associated with significantly enhanced DNMT1 and 5mC amounts at the *C22orf2* promoter in MCFs from bone marrow samples compared to normal control ($P < 0.05$ or less). (B) CBY1 protein down-modulation was associated with a significant increment of β catenin and of its target gene cyclin D1 ($P < 0.05$ or less). Actin was used as control for protein loading. See legend to Figure 1 for other abbreviations.

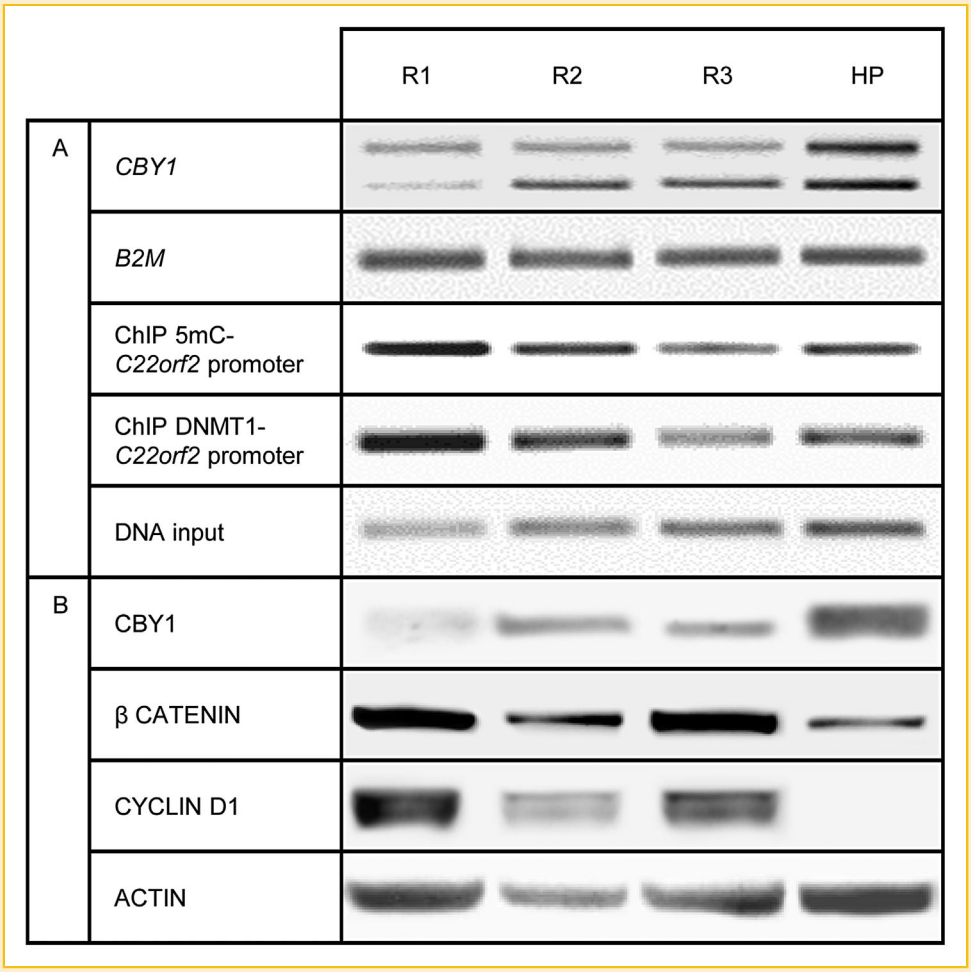

Fig. 3. DNMT1 recruitment leading to hyper-methylation of *C22orf2* promoter and CBY1 transcript reduction at the moment of clinical diagnosis in CML patients who developed IM resistance during therapy. (A) At the moment of clinical diagnosis, MCFs from bone marrow samples of all three CML patients who developed BCR-ABL1-independent resistance to IM (R1 through R3) exhibited a significant CBY1 transcript reduction ($P < 0.05$ or less) compared to normal control (HP), associated with a significant increment of 5mC content and DNMT1 recruitment at the *C22orf2* promoter. (B) CBY1 down-modulation was associated with a significant increment of β catenin and its target gene cyclin D1 ($P < 0.01$ or less). See legends to Figures 1 and 2 for details and abbreviations.

antagonist encoded by C22*orf*2 (close to the BCR breakpoint on chromosome 22q11) is a further component of β catenin signalling in CML hematopoietic progenitors and LSC [Takemaru et al., 2003; Leo et al., 2013]. CBY1 reduction associated with *BCR-ABL1* TK is partly contingent upon transcriptional events and mediated by hyper-methylation of a promoter region critical for gene transcription [Leo et al., 2013; Mancini et al., 2013]. Our results provided evidence of the role of DNMT1 enhanced recruitment in C22*orf*2 promoter hyper-methylation leading to CBY1 down-regulation associated with the *BCR-ABL1 TK* (Fig. 1A). Accordingly, CBY1 transcriptional induction was also promoted by 5-Aza-CdR, which degrades DNMT1 without affecting the *BCR-ABL1* protein enzymatic activity, through C22*orf*2 promoter de-methylation (Fig. 1B) [Ghoshal et al., 2005]. However, DNMT1 release from the C22*orf*2 promoter, resulting in its de-methylation, persisted longer than CBY1 transcriptional induction and paralleled the inhibition of p210 BCR-ABL enzymatic activity by IM (Fig. 1). The findings support the participation of additional events in chromatin epigenetic modification(s) driving β catenin activation in CML. Such events may encompass the histone H3 de-methylation at critical residues for chromatin accessibility to transcription factors and 5mC hydroxylation by Ten-Eleven-Translocation 2 (TET2) dioxygenase associated with the *BCR-ABL1* rearrangement, and may concur with DNMT1 to regulate CBY1 transcription in CML [Mancini et al., 2011, 2012]. Our results support the putative role of CBY1 and DNMT1 in the *BCR-ABL1*-driven transformation of hematopoiesis, already advanced in lung cancers [Xu et al., 2011].

The IM-resistant phenotype in *BCR-ABL1* + CML and chronic eosinophilic leukemia have been associated with DNA hyper-methylation-mediated silencing of the phosphatase and tensin homolog deleted on chromosome ten (PTEN) gene [Nishioka et al., 2010, 2011]. The above showed correlation between DNMT1-driven methylation status of the C22*orf*2 promoter and CBY1 expression following *BCR-ABL1* inhibition in response to IM addressed further

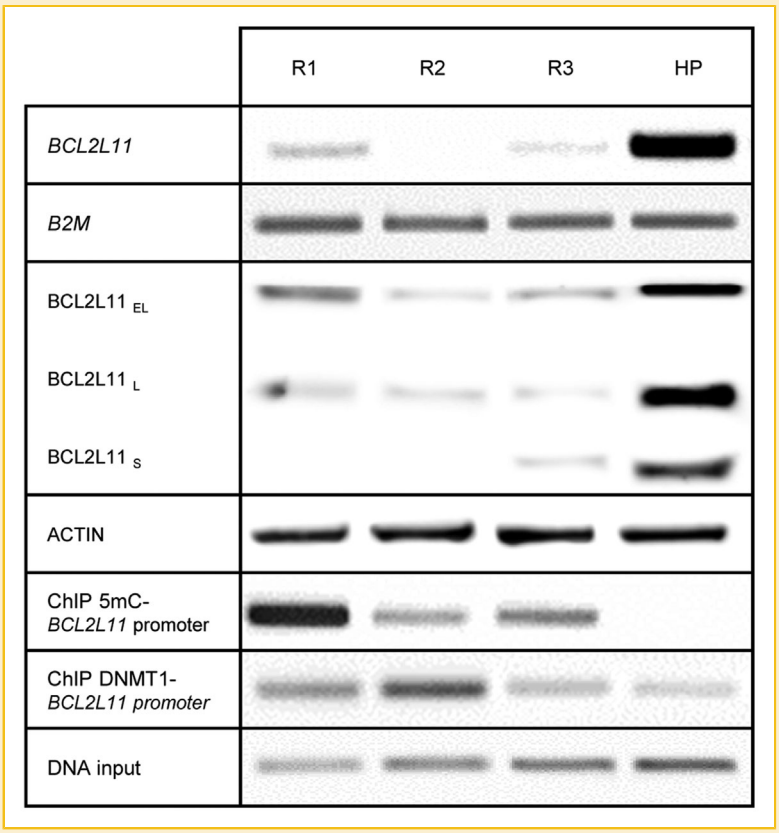

Fig. 4. DNMT1 recruitment leading to hyper-methylation of BCL2-like11 promoter and transcript reduction at the moment of clinical diagnosis in CML patients who developed IM resistance during therapy. DNMT1 and 5mC amounts at BCL2-like11 promoter region encompassing an ATG internal region of 358 bp encompassing nucleotides -268 to +90 critical for gene transcription correlated with its transcript and protein significant reduction ($P < 0.05$ or less) in all three IM-resistant CML patients (R1 through R3). See legends to Figures 1 and 2 for abbreviations.

investigation, aimed to determine the prognostic value of such epigenetic trait in terms of CML responsiveness to IM in vivo. To this purpose, we integrated our research with the DNMT1-dependent methylation status of *BCL2-like11,* a crucial tumour suppressor gene for prognosis of IM-treated CML patients [Kuroda et al., 2006; San José-Eneriz et al., 2009]. *BCL2-like11* dysfunction either due to an intronic deletion polymorphism or to a single nucleotide polymorphism (c465C > T) has been associated with CML resistance to IM [Ng et al., 2012; Augis et al., 2013; Katagiri et al., 2013]. Although the patient cohort included in our study was quite small, our results support that DNMT1-induced hyper-methylation of C22*orf*2 and *BCL2-like11* promoters at the moment of clinical diagnosis was associated with gene transcriptional down-modulation, but not correlated with the outcome of CML resistance to IM during treatment (Figs. 2–4; Leo et al., 2012).

C22*orf*2 and *BCL2-like11* are neither the sole nor the most important genes whose expression is down-regulated by promoter hyper-methylation. Indeed, the CpG island methylator phenotype is a common event in CML, eventually associated with the disease progression and drug resistance outcome [Janssen et al., 2010; Jelinek et al., 2011; Leo and Martinelli, 2014]. In first instance, it concerns the two genes involved in t (9;22) reciprocal translocation which generates the *BCR-ABL1* gene. The allele-specific de novo methylation of *ABL1* promoter (Pa) nested within the chimeric oncogene progressively increases with the disease progression [Zion et al., 1994; Asimakopoulos et al., 1999; Sun et al., 2001]. It may be one component of *ABL1* tumor suppressor function inactivation. *BCR* promoter methylation correlates with a better response to IM through events likely encompassing *BCR-ABL1* transcription [Koh et al., 2011]. Moreover, *BCR-ABL1*-associated DNA hyper-methylation affects genes coding for transcription factors (JunB, IRF-4, CEBPA, HOXA4, PU.1, TFAP2A, and EBF1), tumor suppressors (SOCS1, PLCD1, DAPK1, PTPRG, and DDIT3), and tumor-associated antigens (PRAME) [Machova et al., 2013; Leo and Martinelli, 2014]. The identification of DNMT1 as a component of DNA hyper-methylation leading to transcriptional down-modulation of C22*orf*2 and *BCL2-like11* tumor suppressor genes involved in BCR-ABL1 leukemogenesis support the use of DNMT1-targeting agents in treatment of advanced phases or resistant to IM CML [Katarjian et al., 2003; Issa et al., 2005; Oki et al., 2007]. However, DNMT1-driven hyper-methylation of C22*orf*2 and *BCL2-like11* in more differentiated CML progenitors at diagnosis are neither correlated with CML prognosis nor predict the disease responsiveness to IM (Figs. 2–4; Leo et al., 2013). Further investigation in LSC is required

to establish whether such epigenetic eventually drives CML clonal evolution towards drug resistance, as other tumor suppressor genes do [Janssen et al., 2010].

ACKNOWLEDGMENTS

E.L. and M.M. equally contributed to the study. They carried out the molecular genetic studies, performed all statistical analyses, and drafted the manuscript. F.C. and G.G. provided clinical details of CML patients included in the study; M.A.S. and G.M. conceived the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the manuscript. M.M. is the recipient of a grant provided by the Umberto Veronesi Foundation. Umberto Veronesi Foundation, E.L.N., Bologna*AIL*, AIRC, PRIN, progetto Regione-Università 2010-12 (L. Bolondi), FP7 NGS-PTL project are acknowledged for financial support. The authors thank Ken-Ichi Takemaru for helpful suggestions.

REFERENCES

Afar DE,McLaghlin J, Sherr CJ, Witte ON, Roussel MF. 1995. Signaling by ABL oncogenes through cyclin D1. Proc Natl Acad Sci USA 92:9540–9544.

Asimakopoulos FA, Shteper PJ, Krichevsky S, Fibach E, Polliack A, Rachmilewitz E, Ben. Neriah Y, Ben-Yehuda D. 1999. ABL1 methylation is a distinct molecular event associated with clonal evolution of chronic myeloid leukemia. Blood 4:2452–2460.

Augis V, Airiau K, Josselin M, Turcq B, Mahon FX, Belloc F. 2013. A single polymorphism in cBIM is associated with a slower achievement of major molecular response in chronic myeloid leukemia treated with imatinib. PLoS ONE 8:e78582.

Chu S, McDonald T, Lin A, Chakraborty S, Huang Q, Snyder DS, Bhatia R. 2011. Persistence of leukemic stem cells in chronic myelogenous leukemia patients in prolonged remission with imatinib treatment. Blood 118: 5565–5572.

Coluccia AM, Vacca A, Dunach M, Mologni L, Redaelli S, Bustos VH, Benati D, Pinna LA, Gambacorti-Passerini C. 2007. Bcr-Abl stabilizes beta-catenin in chronic myeloid leukemia through its tyrosine phosphorylation. EMBO J 26:1456–1466.

Copland M, Hamilton A, Elrick LJ, Baird JW, Allan EK, Jordanides N, Barow M, Mountford JC, Hoyoake TL. 2006. Dasatinib (BMS-354825) targets and earlier progenitor population than imatinib but does not eliminate the quiescent fraction. Blood 107:4532–4539.

Corbin AS, Agarwal A, Loriaux M, Corte s J, Deininger MW, Druker BJ. 2011. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite the inhibition of BCR-ABL activity. J Clin Invest 121:396–409.

Druker BJ, Guilhot F, O′Brien SG, Gathmann I, Kantarjian H, Gattermann N, Deininger MW, Silver RT, Goldman JM, Stone RM, Cervantes F, Hochhaus A, Powell BL, Gabrilove JL, Rousselot P, Reiffers J, Cornelissen JJ, Hughes T, Agis H, Fischer T, Verhoef G, Shepherd J, Saglio G, Gratwohl A, Nielsen JL, Radich JP, Simonsson B, Taylor K, Baccarani M, So C, Letvak L, Larson RA, IRIS Investigators. 2006. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. N Engl J Med 355:2408–2417.

Ghoshal K, Datta J, Majumder S, Bai S, Kutay H, Motiwala T, Jacob ST. 2005. 5-Aza-deoxycytidine induces selective degradation of DNA methyltransferase 1 by a proteasomal pathway that requires the KEN box, bromo-adjacent homology domain, and nuclear localization signal. Mol Cell Biol 25: 4727–4741.

Graham SM, Jorgensen HG, Allan E, Pearson C, Alcorn MJ, Richmond L, Holyoake TL. 2002. Primitive, quiescent Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. Blood 99:319–325.

Hamilton A, Helgason GV, Schemionek M, Zhang B, Myssina S, Allan EK, Nicolini FE, Muller-Tidow C, Bhatia R, Brunton VG, Koschmieder S, Holyoake TL. 2012. Chronic myeloid leukemia stem cells are not dependent on Bcr-Abl kinase activity for survival. Blood 119:1501–1510.

Henderson BR, Fagotto F. 2002. The ins and outs of APC and β-catenin nuclear transport. EMBO Rep 3:834–839.

Holland JD, Klaus A, Garratt AN, Birchmeier W. 2013. Wnt signalling in stem and cancer stem cells. Curr Opin Cell Biol 25:254–264.

Hu Y, Chen Y, Douglas L, Li S. 2009. Beta-Catenin is essential for survival of leukemic stem cells insensitive to kinase inhibition in mice with BCR-ABL-induced chronic myeloid leukemia. Leukemia 23:109–116.

Huang W, Bei L, Eklund EA. 2013. Fas-associated phosphatase 1 (Fap1) influences beta catenin activity in myeloid progenitor cells expressing the Bcr-abl oncogene. J Biol Chem 288:12766–12776.

Huang W, Zhou W, Saberwal G, Konieczna I, Horvath E, Katsoulidis E, Platanias LC, Eklund EA. 2010. Interferon consensus sequence binding protein (ICSBP) decrases beta-catenin activity in myeloid cells by repressing GAS2 transcription. Mol Cell Biol 30:4575–4594.

Iqbal Z, Aleem A, Iqbal M, Naqvi MI, Gill A, Taj AS, Qayyum A, ur-Rehman K, Khalid AM, Shah IH, Khalid M, Haq R, Khan M, Baing SM, Jamil A, Abbas MN, Absar M, Mahmood A, Rasool M, Akhtar T. 2013. Sensitive detection of pre-existing BCR-ABL kinase domain mutations in CD34+ cells of newly diagnosed chronic-phase chronic myeloid leukemia patients is associated with imatinib resistance: Implications in the post-imatinib era. PLoS ONE 8:e55717.

Issa JP, Gharibyan V, Cirtes J, Jelinek J, Morris G, Verstovsek S, Talpaz M, Garcia-Manero G, Kantarjian HM. 2005. Phase II study of low-dose decitabine in patients with chronic myelogenous leukemia resistant to imatinib mesylate. J Clin Oncol 23:3948–3956.

Jamieson CH, Ailles LE, Dvilla SJ, Muijtjens M, Jones C, Zehnder JL, Gotlib J, Li K, Manz MG, Keating A, Sawyers CL, Weissman IL. 2004. Granulacyte-macrphage progenitors as candidate leukemic stem cells in blast-crisis CML. N Engl J Med 351:657–667.

Janssen JJ, Denkers F, Valk P, Cornelissen JJ, Schuurhuis GJ, Ossenkoppele GJ. 2010. Methylation patterns in CD34 positive chronic myeloid leukemia blast crisis cells. Haematologica 95:1036–1037.

Jelinek J, Gharibyan V, Estecio MR, Kondo K, He R, Chung W, Lu Y, Zhang N, Liang S, Kantarjian HM, Cortes JE. 2011. Aberrant DNA methylation is associated with disease progression, resistance to imatinib and shortened survival in chronic myelogenous leukemia. PLoS ONE 6:e22110.

Jorgensen HG, Allan EK, Jordanides NE, Mountford JC, Holyoake TL. 2007. Nilotinib exerts equipotent antiproliferative effects to imatinib and does not induce apoptosis in CD34+ CML cells. Blood 109:4016–4019.

Katagiri S, Umezu T, Ohyashiki JH, Ohyashiki K. 2013. The BCL2L11 (BIM) deletion polymorphism is a possible criterion for discontinuation of imatinib in chronic myeloid leukemia. Br J Haematol 160:269–272.

Katarjian HM, O'Brien S, Cortes J, Giles FJ, Faderl S, Issa JP, Garcia-Manero G, Rios MB, Shan J, Andreeff M, Keating M, Talpaz M. 2003. Results on decitabine (5-aza-2′ deoxycytidine) therapy in 130 patients with chronic myelogenous leukemia. Cancer 98:522–528.

Koh Y, Kim DY, Park SH, Byun HM, Kim I, Yoon SS, Kim KB, Park E, Yang AS, Park S. 2011. Increased BCR promoter DNA methylation status strongly correlates with favorable response to imatinib in chronic myeloid leukemia patients. Oncol Let 2:181–187.

Kuroda J, Puthalakath H, Cragg MS, Kelly PN, Bouillet P, Huang DC, Kimura S, Ottmann OG, Druker BJ, Villunger A, Roberts AW, Strasser A. 2006. Bim and Bad mediate imatinib-induced killing of Bcr/Abl+ leukemic cells, and resistance to their loss is overcome by BH3 mimetic. Proc Natl Acad Sci USA 103:14907–14912.

La Roseée P, Hochhaus A. 2008. Resistance to imatinib in chronic myelogenous leukemia: Mechanisms and clinical implications. Curr Hematol Malig Rep 3:72–79.

Lemoli RM, Salvestrini V, Bianchi E, Bertolini F, Fogli M, Amabile M, Tafuri A, Salati S, Zini R, Testoni N, Rabascio C, Rossi L, Martin-Padura I, Castagnetti F, Marighetti G, Baccarani M, Ferrari S, Manfredini R. 2009. Molecular and functional analysis of the stem cell compartment of chronic myelogenous leukemia reveals the presence of a CD34- cell population with intrinsic resistance to imatinib. Blood 114:5191–5200.

Leo E, Mancini M, Aluigi M, Castagnetti F, Martinelli G, Barbieri E, Santucci MA. 2012. DNA hypermethylation promotes the low expression of pro-apoptotic BCL2L11 associated with BCR-ABL1 fusion gene of chronic myeloid leukemia. Br J Haematol 159:373–379.

Leo E, Mancini M, Aluigi M, Luatti S, Castagnetti F, Testoni N, Soverini S, Santucci MA, Martinelli G. 2013. BCR-ABL1-associated reduction of beta catenin antagonist Chibby 1 in chronic myeloid leukemia. PLoS ONE 8:e81425.

Leo E, Martinelli G. 2014. DNA Methylation in Chronic Myeloid Leukemia. J Mol Genet Med 8:118.

Li FQ, Mofunanya A, Harris K, Takemaru K. 2008. Chibby cooperates with 14–3–3 to regulate beta-catenin subcellular distribution and signalling activity. J Cell Biol 181:1141–1154.

Machova Polakova K, Koblihova J, Stopka T. 2013. Role of epigenetics in chronic myeloid leukemia. Curr Hematol Malig Rep 8:28–36.

Mancini M, Aluigi M, Leo E, Marcozzi C, Castagnetti F, Barbieri E, Santucci MA. 2011. Histone H3 covalent modifications driving response of BCR-ABL1+ cells sensitive and resistant to imatinib to Aurora kinase inhibitor MK-0457. Br J Haematol 156:265–268.

Mancini M, Leo E, Takemaru K, Campi V, Borsi E, Castagnetti F, Gugliotta G, Santucci MA, Martinelli G. 2013. Chibby drives beta catenin cytoplasmic accumulation leading to activation of the unfolded protein response in BCR-ABL1+ cells. Cell Signal 25:1820–1827.

Mancini M, Petta S, Iacobucci I, Salvestrini V, Barbieri E, Santucci MA. 2010. Zinc-finger transcription factor slug contributes to the survival advantage of chronic myeloid leukemia cells. Cell Signal 22:1247–1253.

Mancini M, Veljkovic N, Leo E, Aluigi M, Borsi E, Galloni C, Iacobucci I, Barbieri E, Santucci MA. 2012. Cytoplasmic compartmentalization by Bcr-Abl promotes TET2 loss-of-function in chronic myeloid leukemia. J Cell Biochem 113:2765–2774.

McWhirter JR, Galasso DL, Wang JY. 1993. A coiled-coil oligomerization domain of Bcr is essential for the transforming function of Bcr-Abl oncoproteins. Mol Cell Biol 13:7587–7595.

Minami Y, Stuart SA, Ihawa T, Jiang Y, Banno A, Hunton IC, Young DJ, Naoe T, Murre C, Jamieson CH, Wang JY. 2008. BCR-ABL-transformed GMP as myeloid stem cells. Proc Natl Acad Sci USA 105:17967–17972.

Ng KP, Hillmer AM, Chuah CT, Juan WC, Ko K, Teo AS, Ariyaratne PN, Takahashi N, Sawada K, Fei Y, Soh S, Lee WH, Huang JW, Allen JC, Woo XY, Nagarajan N, Kumar V, Thalamuthu A, Poh WT, Ang AL, Mya HT, How GF, Yang LY, Koh LP, Chowbay B, Chang CT, Nadarajan VS, Chng WJ, Than H, Lim LC, Goh YT, Zhang S, Poh D, Tan P, Seet JE, Ang MK, Chau NM, Ng QS, Tan DS, Soda M, Isobe K, Nöthen MM, Wong TY, Shahab A, Ruan X, Cacheux-Rataboul V, Sung WK, Tan EH, Yatabe Y, Mano H, Soo RA, Chin TM, Lim WT, Ruan Y, Ong ST. 2012. A common BIM deletion polymorphism mediates intrinsic resistance and inferior responses to tyrosine kinase inhibitors in cancer. Nat Med 18:521–528.

Nishioka C, Ikezoe T, Yang J, Udaka K, Yokoyama A. 2011. Imatinib causes epigenetic alterations of PTEN gene via upregulation of DNA methyltransferases and polycomb group proteins. Blood Cancer J 1:e48.

Nishioka C, Ikezoe T, Yang J, Yokoyama A. 2010. Long-term exposure of leukemia cells to multi-targeted tyrosine kinase inhibitor induces activations of AKT, ERK and STAT5 signaling via epigenetic silencing of the PTEN gene. Leukemia 24:1631–1640.

Oki Y, Kantarjian HM, Gharibyan V, Jones D, O'Brien S, Verstovsek S, Cortes J, Morris GM, Garcia-Manero G, Issa JP. 2007. Phase II study of low-dose decitabine in combination with imatinib in patients with accelerated or myeloid blastic phase of chronic myelogenous leukemia. Cancer 109:899–906.

Roychowdhury S, Talpaz M. 2011. Managing resistance in chronic myeloid leukemia. Blood Rev 25:279–290.

San José-Eneriz E, Agirre X, Jimémez-Velasco A, Cordeu L, Martìn, Arqueros V, Gárate L, Fresquet V, Cervantes F, Martìnez-Climent JA, Heiniger A, Torres A, Pòsper F, Roman-Gomez J. 2009. Epigenetic down-regulation of BIM expression is associated with reduced optimal responses to imatinib treatment in chronic myeloid leukemia Eur J Cancer 45:1877–1889.

Sokal JE, Gomez GA, Baccarani M, Tura S, Clarkson BD, Cervantes F, Rozman C, Carbonell F, Anger B, Heimpel H. 1988. Prognostic significante of additional cytogenetic abnormalities at diagnosis of Philadelphia chromosome-positive chronic granulocytic leukemia. Blood 72:294–298.

Sun B, Jiang G, Zaydan MA, La Russa VF, Safah H, Ehrlich M. 2001. ABL1 promoter methylation can exist independently of BCR-ABL transcription in chronic myeloid leukemia hematopoietic progenitors. Cancer Res 61:6931–6937.

Takemaru K, Yamaguchi S, Lee YS, Zhang Y, Carthew RW, Moon RT. 2003. Chibby, a nuclear beta-catenin-associated antagonist of the Wnt/Wingless pathway. Nature 422:905–909.

Xu HT, Li OC, Dai SD, Xie XM, Liu DI, Wang EH. 2011. The expression patterns and correlations of chibby, β-catenin, and DNA methyltransferase-1, and their clinicopathological significance in lung cancers. APMIS 119:750–758.

Zhao C, Blum J, Chen A, Kwon HY, Jung SH, Cook JM, Lagoo A, Reya T. 2007. Loss of beta-catenin impairs the renewal of normal and CML stem cells in vivo. Cancer Cell 12:528–541.

Zion M, Ben-Yehuda D, Avraham A, Cohen O, Wetzler M, Melloul D, Ben-Neriah Y. 1994. Progressive de novo DNA methylation at the bcr-abl locus in the course of chronic myeloid leukemia. Proc Natl Acad Sci USA 91:10722–10726.