Published in final edited form as:

Leuk Res. 2009 October ; 33(10): . doi:10.1016/j.leukres.2009.03.044.

DOWNREGULATION OF *JUNB* mRNA EXPRESSION IN ADVANCED PHASE CHRONIC MYELOGENOUS LEUKEMIA

Koyu Hoshino¹, Alfonso Quintás-Cardama¹, Jerald Radich², Hongyui Dai³, Hui Yang¹, and
Guillermo Garcia-Manero¹

¹Department of Leukemia, University of Texas M.D. Anderson Cancer Center, Houston, TX

²Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA

³Rosetta Inpharmatics, Seattle, WA

Abstract

JUNB inactivation in transgenic mice results in a myeloproliferative disorder that closely resembles human chronic myelogenous leukemia (CML). It has been reported that downregulation of *JUNB* expression is a universal phenomenon in patients with CML due aberrant DNA methylation of its promoter. Based on this, we studied methylation and gene expression levels of *JUNB* in CML. We analyzed the methylation status of the *JUNB* gene in 6 cell lines and in 102 patients with CML using several bisulfite PCR assays. *JUNB* expression was analyzed using real-time PCR and gene expression profiling. *JUNB* methylation was not observed in any of the cell lines studied, and only in 3% of patients with CML. Despite the lack of *JUNB* methylation, *JUNB* was expressed at low levels both in CML cell lines (median dCT −6.86; range −5.87 to −9.61), and in patients with CML in blastic phase (BP) (median dCT −3.95; range −1.48 to −6.29) ($p=0.002$). Finally, we evaluated *JUNB* expression in 82 additional patients with CML by gene expression arrays. We found that *JUNB* was significantly downregulated in advanced phase CML in contrast to chronic phase CML (median log ratio difference in expression = 0.53). Overall, our results indicate that *JUNB* expression is downregulated in advanced phase CML through a mechanism independent from DNA methylation.

Keywords

JUNB; DNA methylation; chronic myelogenous leukemia

© 2009 Elsevier Ltd. All rights reserved.

Corresponding Author: Guillermo Garcia-Manero, MD, Department of Leukemia, University of Texas MD Anderson Cancer Center, Box 428, 1515 Holcombe Blvd, PO BOX 301402, Houston, TX 77230-1402, Phone: 713 745 3428, Fax: 713 794 4297, ggarciam@mdanderson.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of Interest

There was no conflict of interest for any of the authors.

Contributions

KY performed all the experiments, analyzed the data and wrote the manuscript. A Q-C wrote the manuscript and analyzed the data. HD and HY performed research. JR wrote manuscript, and analyzed data. G G-M designed and funded the study, wrote the manuscript and analyzed the data.

Introduction

The transcription factor activator protein 1 (AP-1) comprises a group of hetero and homodimeric basic region-leucine zipper (bZIP) proteins that belong to the JUN (c-JUN, JUNB, JUND), FOS (c-FOS, FOSB, FRA-1 and FRA-2), MAF (c-MAF, MAFB, MAFA, MAFK/F/K and NRL) and ATF (ATF2, LRF1/ATF3, B-ATF, JDP1, JDP2) subfamilies (1, 2). Although structurally similar to the other *JUN* genes, *JUNB* exhibits weaker DNA-binding affinity to AP-1 DNA recognition elements and has been shown to exert both transactivator and transrepressor effects depending on the promoter context and on the heterodimerization partner (3, 4). The knockout of *JUNB* in mice results in early embryonic lethality, while constitutive overexpression of *JUNB* under the control of the human *Ubiquitin-C* promoter (*Ubi*) has no major consequences in *Ubi-JUNB* transgenic mice (5). *JUNB* suppresses cell proliferation by direct transcriptional activation of the cyclin-dependent kinase inhibitor p16^{INK4a} (6), repression of cyclin D1, a component of the G₁ cyclin–cyclin-dependent kinase complex (7), and regulation of the expression of BCL-2 and BCLx (6, 8). The balance between the antagonistic biological effects exerted by *JUNB* and c-JUN during mitosis is fundamental for the regulation of cell cycle progression (7). Furthermore, *JUNB* is a crucial transcriptional regulator of myelopoiesis and its expression plays a role in the initiation, progression and maintenance of the myeloid differentiation program (8, 9). *JUNB* is constitutively expressed in human peripheral blood mature granulocytes and its expression is strongly induced following terminal differentiation of bone marrow myeloid precursors and established myeloid cell lines (9, 10). *JUNB* overexpression results in suppression of RAS- and SRC-induced tumor growth *in vivo*, suggesting a role of *JUNB* as a tumor suppressor gene. Moreover, c-JUN binds to and represses the promoter of the tumor suppressor gene *p53*, followed by downregulation of its target gene the CDK inhibitor *p21*, whereas lack of expression of *c-JUN* upregulates *p53* and *p21* expression and accelerates cell proliferation (11). Several reports have shown that *JUNB* inhibits c-JUN-mediated transactivation and transforming activity (11–13) and promotes growth arrest and differentiation (14–16).

Further *in vivo* evidence supporting the function of *JUNB* as tumor suppressor gene has been provided by *JUNB*^{−/−} *Ubi-JUNB* transgenic mice generated by intercrossing *JUNB*^{+/−} animals carrying the *Ubi-JUNB* transgene (8). These animals develop a transplantable myeloproliferative disorder (MPD) that strikingly resembles CML, including progression to blastic phase (BP) (8). Notably, *JUNB* inactivation specifically expands the number of long-term hematopoietic stem cells (LT-HSC) and granulocyte/macrophage progenitors resulting in a chronic MPD (17). Inactivation of *JUNB* expression has been previously reported in some human CML patients although the mechanism whereby this gene is inactivated has remained elusive (18). A report in 32 patients with CML indicated that most of the CpG sites in the promoter area of *JUNB* were methylated, suggesting that this epigenetic mechanism was responsible for *JUNB* silencing in 100% of the patients evaluated (19). In view of the implications of *JUNB* silencing in murine models, and the aforementioned report suggesting universal methylation-induced *JUNB* inactivation in a small cohort of patients, we evaluated the incidence of *JUNB* aberrant DNA methylation in CML-derived cell lines and in 102 patients with CML. We also determined *JUNB* mRNA expression by reverse transcription polymerase chain reaction (RT-PCR) in 27 patients with CML and validated these results analyzing *JUNB* gene expression in a separate cohort of 82 patients with CML by cDNA microarray analysis.

Materials and methods

Analysis of DNA methylation and cell lines

DNA methylation was analyzed using 3 different bisulfite polymerase chain reaction (PCR) assays mapping 2 different genomic areas in the proximity of the *JUNB* transcription start site (Figure 1A). Region 1 was the area originally studied by Yang *et al* (19). This region was analyzed using the same methylation specific PCR (MSP) assay used by Yang *et al* (19), and also by a second bisulfite PCR assay, developed by us, using the combined bisulfite PCR restriction analysis (COBRA) (20) (figure 1A). To further study the promoter region of *JUNB*, we developed a second COBRA assay mapping region 2 (figure 1A). Bisulfite treatment of DNA (and PCR conditions used) have been previously described (21). Bisulfite-modified DNA was amplified by PCR using primer sets designed specifically for the promoter region of *JUNB* gene. Characteristics of the primers are shown in Table 1. In selected cases, methylation was analyzed also using bisulfite sequencing as previously described (21).

The following cell lines were studied for DNA methylation: K562, K562R, BV173, BV173R, Raji and HL-60. These cell lines were provided by Drs N. Donato and M. Beran (MD Anderson Cancer Center) and were maintain following standard conditions.

Gene expression analysis in samples analyzed for DNA methylation

The levels of *JUNB* mRNA in cell lines and patients with CML previously studied for DNA methylation status were studied. For this analysis, the following cell lines were used: K562, K562R, BV173, BV173R, Raji and HL-60. mRNA expression was analyzed using commercially available primers (Applied Biosystems, Foster City, CA) and the conditions recommend by the manufacturer for real-time (RT) PCR using an ABI7000 sequencer (Applied Biosystems, Foster City, CA). Because *JUNB* has only one exon, RNA was treated with DNAase I prior to cDNA conversion to prevent false positive amplification arising from DNA contamination of the sample mRNA.

Analysis of *JUNB* mRNA expression using cDNA arrays

To further determine the expression levels of *JUNB* mRNA in patients with CML, a second cohort of patients with CML previously studied by ink-jet oligonucleotide array technology gene expression arrays was examined (22). RNA amplification, labeling and hybridization to hu25k ink jet DNA microarrays have been previous described (23, 24). These CML samples were examined for *JUNB* expression comparing the expression level of *JUNB* in each CML sample to the *JUNB* expression in the pool of 200 samples from patients with CP-CML.

Results

Frequency of *JUNB* DNA methylation in CML

JunB methylation was studied in the following cell lines: K562, K562R, BV173, BV173R, Raji and HL-60. Aberrant *JUNB* methylation was not observed in any of the cell lines analyzed by any of the 3 methods used here (data not shown). Next, we analyzed the methylation status of the *JUNB* region 1 in 62 patients with CML using the original MSP assay described by Yang *et al* (19). Of these patients 55 were in chronic phase (CP), 3 in accelerated phase [AP], and 4 (BP). Methylation of this region was not observed in any of these patients [FIGURE 1B]. We then extended this analysis to a total of 102 patients (including the original 62 patients), using the COBRA assay in region-1. Using this assay, *JUNB* methylation was observed in 3 (3%) patients [FIGURE 1C]. Interestingly, all these 3 patients had CML in AP. Methylation results were confirmed in these 3 patients using bisulfite sequencing and in cell lines (FIGURE 2). Because DNA methylation can be

heterogeneous, we analyzed the methylation status of a region upstream of the transcription start site (region-2) in 35 patients (13 in CP, 11 in AP, and 11 in BP) using a COBRA assay. Methylation was not observed in any of these patients (figure 1C). Finally, in view that transgenic mice lacking *JUNB* developed a MPD closely resembling human CML independent of the presence of *BCR-ABL1* involvement (12), we studied 9 patients with Philadelphia (Ph) chromosome-negative *BCR-ABL1*-negative CML. Using the region 1 MSP assay, we failed to demonstrate promoter DNA methylation in *JUNB* (FIGURE 3). No methylation was detected with either of the COBRA assays mapping regions 1 or 2 in these patients with Ph- CML (data not shown). The characteristics of CML patients in our cohort have been previously described and were representative of patients treated at M. D. Anderson Cancer Center (25). All samples were obtained following institutional guidelines.

JUNB expression in CML

Because DNA methylation is not the only mechanism of gene expression regulation, the above results did not exclude the possibility that *JUNB* could be downregulated in patients with CML via a DNA methylation independent mechanism. To study this possibility, we analyzed the levels of *JUNB* mRNA expression in CML-derived cell lines and in patient samples by using RT-PCR. The same cell lines analyzed for DNA methylation were used for expression analysis. Using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as control, the dCT of *JUNB* in K562 was -9.61. *JUNB* expression levels were also analyzed in K562R, BV173 and BV173R. The dCT for these three cell lines ranged from -4.7 to -6.8. These results indicate that *JUNB* is expressed at very low levels in CML derived cell lines independently of the methylation status of *JUNB* gene (FIGURE 4A). We next studied the levels of *JUNB* expression in 27 patients with CML (10 in CP, 9 in AP, and 8 in BP), 6 patients with AML and 6 with Ph+ ALL and 5 healthy volunteers by RT-PCR and the 9 patients with Ph- CML (FIGURE 4B). The median dCT of patients in CP was -1.78 (range ± 1.56), -2.2 (range ± 2.65) in AP, -4.04 (range ± 2.65) in BP, -1.88 (range ± 0.21) in healthy volunteers and 1.56 (range ± 1.01) in AML and non-detectable in ALL. No differences in *JUNB* levels were observed between healthy volunteers, and patients in CP ($p=0.002$). In contrast, lower *JUNB* levels were observed in patients with CML in BP compared to CML in CP ($p=0.002$). These results indicate that *JUNB* levels are very low in CML-derived cell lines and that *JUNB* expression gradually declines during progression to advanced phases of CML, with the lowest *JUNB* levels obtained in patients with BP-CML. Of interest, *JUNB* levels in Ph-CML were higher than those observed in Ph+ CML (FIGURE 4B).

To validate these results, we analyzed *JUNB* gene expression in 82 CML cases previously studied by gene expression arrays (42 in CP, 9 in AP, and 31 in BC). Each individual CML sample in these studies was compared to a pool of mRNA from 200 CP-CML cases. As shown in FIGURE 5, results of gene expression arrays confirmed a progressive loss of *JUNB* expression in patients with advanced phases of CML. The expression of *JUNB* was heterogeneous in samples obtained from patients with CP-CML (left panel figure 5A), but patients with AP-CML exhibited decreased expression of *JUNB* when compared with CP-CML. Moreover, nearly all patients in BP-CML exhibited decreased *JUNB* expression. In fact, the median decrease expression in BP-CML samples compared to CP-CML was 0.53 logs (ANOVA $p < 0.0001$).

Finally, we analyzed the effect of imatinib based therapy in 9 patients with CML and observed that the expression of *JUNB* increases in patients with CP-CML during therapy with the *BCR-ABL1* kinase inhibitor imatinib, suggesting the expression of this gene may be regulated by the activity of this kinase (FIGURE 6).

Discussion

JUNB modulates myelopoiesis and is a potential tumor suppressor gene in mice (8). Adult *JUNB*^{-/-} *Ubi-JUNB* mice developed a transplantable MPD with complete penetrance by 11 months of age, which coincides with a myeloid-specific and age-dependent loss of *JUNB* expression. This disease strikingly resembles CML, both clinically and morphologically. In fact, 16% of these mice experience progression to BP, thereby recapitulating the natural course of human CML (8). Herein, we demonstrate by two different analytical methods that *JUNB* mRNA expression is downregulated in advanced phase CML and this occurrence is not mediated by aberrant DNA methylation. This is of importance because inactivation of *JUNB* expression in LT-HSC, but not in cells with a higher degree of hematopoietic commitment, invariably leads to leukemogenic stem cell expansion and development of a transplantable MPD similar to CML (17). Conversely, enforced expression of *JUNB* in HSC through lentiviral transduction causes stem cell loss (17). It has been reported that *JUNB* expression is impaired in peripheral blood cells of patients with *BCR-ABL1*-positive CML (18). The specific factors leading to downregulation of *JUNB* remain largely unknown. It has been speculated that *JUNB* could be downregulated due to epigenetic silencing of the *JUNB* gene promoter (8). DNA hypermethylation of CpG islands in the proximity of gene promoters is a well-characterized epigenetic phenomenon (26) linked to transcriptional silencing of tumor-suppressor genes in both solid tumors (27–28) and leukemia (29–32), thus leading to carcinogenesis (32–34). Several reports have emphasized the importance of methylation-induced gene silencing in the pathogenesis of CML (34–36). Yang *et al* have reported on the *JUNB* expression status of 32 patients with CML (21 in CP and 11 in BP) (19). *JUNB* was downregulated in all patients but no mutations were demonstrated within the *JUNB* gene-coding region. Interestingly, most of the CpG sites were methylated in the promoter area of the *JUNB* gene, suggesting that *JUNB* inactivation was due to methylation of the promoter region of the *JUNB* gene (19). In stark contrast, our results indicate that *JUNB* methylation is rare in patients with CML. In fact, *JUNB* methylation was observed in none of the *BCR-ABL1*-positive cell lines analyzed, and only 3 (3%) of 102 patients with CML (and none of 9 patients with Ph-negative CML) exhibited a significant degree of methylation in the promoter area of *JUNB*. Of note, these 3 patients had AP-CML. Overall, these results indicate that downregulation of *JUNB* in patients with CML is modulated by mechanisms of gene expression different from DNA methylation and that *JUNB* methylation is not regulated by *BCR-ABL1*. In addition, when RT-PCR analysis was used to determine *JUNB* mRNA expression, no significant differences were observed between healthy individuals and patients with CP-CML. However, *JUNB* expression declined in a progressive manner with advanced CML disease. These results were confirmed by gene expression microarray analysis, further strengthening the association between *JUNB* downregulation and CML phase progression. Interestingly, the expression of *JUNB* increased in patients with CP-CML during imatinib therapy suggesting that its expression may be regulated by the activity of BCR-ABL1 kinase.

Several factors could account for the striking disparity between our results and those previously reported (19). First, part of this discrepancy may be related to methodological aspects, particularly regarding the sensitivity of the MSP assay and the cohort size used by the Yang *et al* (19). We circumvented this potential bias by performing methylation analysis by two different methylation assays (MSP and COBRA) in a significantly larger cohort of patients. Secondly, differences in DNA methylation patterns have been observed among different ethnic populations and geographic areas (37, 38). However, a difference in methylation levels close to 100% cannot be explained solely on the basis of ethnic differences between U.S. and Asian CML patient populations, particularly since the *BCR-ABL1* oncogene that drives CML pathogenesis, is universally found in all patients independently of the ethnic background. Finally, DNA promoter methylation has been

dynamically linked to histone deacetylase activity (39). In fact, these two epigenetic processes appear to function as synergistic layers for the silencing of genes in cancer (39). Hence, it would be reasonable to ascribe part of the *JUNB* downregulation seen in our patients to histone deacetylase activity in the absence of significant DNA methylation. Further analysis of chromatin code will be needed to validate this issue in CML.

BCR-ABL1 is regarded as sufficient and necessary for the pathogenesis of CML (40–42). Imatinib mesylate, a targeted BCR-ABL1 kinase inhibitor, is associated with a complete cytogenetic response rate of 80%–90% in patients with early CP-CML (43). However, even patients who achieve major molecular responses by RT-PCR, experience recurrence of their disease when therapy with imatinib is interrupted, suggesting the presence of residual disease below the threshold of detection of currently employed molecular techniques. This has been attributed to persistence of innately imatinib-insensitive *BCR-ABL1*-positive quiescent cells. It has been shown that approximately 75% of LT-HSCs are quiescent in G₀ phase (44) and that CML may be initiated by transforming events occurring in this subset of cells. *JUNB* regulates the numbers of LT-HSC and granulocyte/macrophage progenitors and knockout mice develop a MPD similar to human CML when devoid of *JUNB* (8, 17). It is conceivable that the pathogenesis of CML may be primarily triggered by downregulation of *JUNB* in LT-HSCs independently of *BCR-ABL1* expression, which might only be required in CML cases arising at the myelomonocytic stage of differentiation. Whether this model translates into human CML pathogenesis warrants further investigation, but provides a model whereby the failure of imatinib in eradicating quiescent leukemic stem cells can be explained by the absence or decreased levels of *BCR-ABL1* expression. Elucidating the determinants of *JUNB* downregulation might facilitate the design of novel targeted therapies for CML. Indeed one of the limitations of this manuscript is that we do not validate the mechanism of *JUNB* down regulation.

In summary, our results indicate that *JUNB* downregulation is a DNA methylation-independent phenomenon encountered in CML only in advanced phases. The mechanisms leading to *JUNB* downregulation and the implications of this occurrence in CML pathogenesis and progression, as well as its relation with prognosis and resistance to therapy warrant further study. The answer to these questions may facilitate the development of novel therapeutic approaches for patients with CML.

Acknowledgments

This work was supported in part by an American Society of Clinical Oncology Career Development Award, the Physician-Scientist Program from the University of Texas MD Anderson Cancer Center, and CA-100067 from the NCI (to G G-M); and CA-85053 from the National Cancer Institute (JR).

References

1. Angel P, Karin M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochem Biophys Acta*. 1991; 1072:129–157. [PubMed: 1751545]
2. Shaulian E, Karin M. AP-1 as a regulator of cell life and death. *Nat Cell Biol*. 2002; 4:E131–E136. [PubMed: 11988758]
3. Deng T, Karin M. Jun B differs from c-Jun in its DNA-binding and dimerization domains, and represses c-Jun by formation of inactive heterodimers. *Genes Dev*. 1993; 7:479–490. [PubMed: 8383624]
4. Hsu JC, Cressman DE, Taub R. Promoter-specific transactivation and inhibition mediated by JunB. *Cancer Res*. 1993; 53:3789–3794. [PubMed: 8339292]
5. Schorpp-Kistner M, Wang ZQ, Angel P, et al. JunB is essential for mammalian placentation. *EMBO J*. 1999; 18:934–948. [PubMed: 10022836]

6. Passegue E, Wanger EF. JunB suppresses cell proliferation by transcriptional activation of p16^{INK4a} expression. *EMBO J.* 2000; 19:2969–2979. [PubMed: 10856241]
7. Bakiri L, Lallemand D, Bossy-Wetzel E, et al. Cell cycle-dependent variations in c-Jun and JunB phosphorylation: a role in the control of cyclin D1 expression. *EMBO J.* 2000; 19:2056–2068. [PubMed: 10790372]
8. Passegue E, Jochum W, Schorpp-Kistner M, Möhle-Steinlein U, et al. Chronic myeloid leukemia with increased granulocyte progenitors in mice lacking JunB expression in the myeloid lineage. *Cell.* 2001; 104:21–32. [PubMed: 11163237]
9. Lord KA, Abdollahi A, Hoffman-Liebermann B, et al. Proto-oncogenes of the fos/jun family of transcription factors are positive regulators of myeloid differentiation. *Mol Cell Biol.* 1993; 13(2): 841–851. [PubMed: 8423806]
10. Mollinedo F, Vaquerizo MJ, Naranjo JR. Expression of c-jun, jun B and jun D proto-oncogenes in human peripheral-blood granulocytes. *Biochem J.* 1991; 273(Pt 2):477–9. [PubMed: 1899335]
11. Schreiber M, Kolbus A, Piu F, et al. Control of cell cycle progression by c-Jun is p53 dependent. *Gene Dev.* 1999; 13:607–619. [PubMed: 10072388]
12. Chiu R, Angel P, Karin M. Jun-B differs in its biological properties from and is a negative regulator of, c-Jun. *Cell.* 1989; 59:979–986. [PubMed: 2513128]
13. Schutte J, Viallet J, Nau M, et al. jun-B inhibits and c-fos stimulates the transforming and trans-activating activities of c-Jun. *Cell.* 1989; 59:987–97. [PubMed: 2513129]
14. Melamed D, Resnitzky D, Haimov I, et al. Interleukin 6 induces DNA binding activity of AP1 in M1 myeloblastic cells but not in a growth resistant cell derivative. *Cell Growth Differ.* 1993; 4:689–697. [PubMed: 8398910]
15. Schlingensiepen K, Schlingensiepen R, Kunst M, et al. Opposite functions of jun-B and c-Jun in growth regulation and neuronal differentiation. *Dev Genet.* 1993; 4:305–312. [PubMed: 8222345]
16. Li B, Tournier C, Davis RJ, et al. Regulation of IL-4 expression by the transcription factor JunB during T helper cell differentiation. *EMBO J.* 1999; 18:420–432. [PubMed: 9889198]
17. Passegue E, Wagner EF, Weissman IL. JunB deficiency leads to a myeloproliferative disorder arising from hematopoietic stem cells. *Cell.* 2004; 119(3):431–443. [PubMed: 15507213]
18. Bruchova H, Borovanova T, Klamova H, et al. Gene expression profiling in chronic myeloid leukemia patients treated with hydroxyurea. *Leuk Lymphoma.* 2002; 43(6):1289–95. [PubMed: 12152998]
19. Yang MY, Liu TC, Chang JG, Lin PM, Lin SF. JunB gene expression is inactivated by methylation in chronic myeloid leukemia. *Blood.* 2003; 101:3205–3211. [PubMed: 12506033]
20. Xiong Z, Laird PW. COBRA: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Res.* 1997; 25:2532–2534. [PubMed: 9171110]
21. Shen L, Toyota M, Kondo Y, et al. Aberrant DNA methylation of p57KIP2 identifies a cell-cycle regulatory pathway with prognostic impact in adult acute lymphocytic leukemia. *Blood.* 2003; 101:4131–4136. [PubMed: 12586619]
22. Radich JP, Dai H, Mao M, et al. Gene expression changes associated with progression and response in chronic myeloid leukemia. *Proc Natl Acad Sci U S A.* 2006; 103(8):2794–2799. [PubMed: 16477019]
23. Hughes TR, Mao M, Jones AR, et al. Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. *Nat Biotechnol.* 2001; 19:342–347. [PubMed: 11283592]
24. Van 't Veer LJ, Dai H, van de Vijver MJ, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature.* 2002; 415:530–536. [PubMed: 11823860]
25. Issa JP, Kantarjian H, Mohan A, et al. Methylation of the ABL1 promoter in chronic myelogenous leukemia: lack of prognostic significance. *Blood.* 1999; 93:2075–2080. [PubMed: 10068681]
26. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet.* 2002; 3:415–428. [PubMed: 12042769]
27. Herman JG, Latif F, Weng Y, et al. Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. *Proc Natl Acad Sci U S A.* 1994; 91:9700–9704. [PubMed: 7937876]

28. Dong SM, Kim H-S, Rha S-H, Sidransky D. Promoter hypermethylation of multiple genes in carcinoma of the uterine cervix. *Clin Cancer Res*. 2001; 7:1982–1996. [PubMed: 11448914]
29. Claus R, Lubbert M. Epigenetic targets in hematopoietic malignancies. *Oncogene*. 2003 Sep 29; 22(42):6489–96. [PubMed: 14528273]
30. Melki JR, Clark SJ. DNA methylation changes in leukaemia. *Semin Cancer Biol*. 2002; 12(5):347–57. [PubMed: 12191634]
31. Issa JP, Baylin SB, Herman JG. DNA methylation changes in hematologic malignancies: biologic and clinical implications. *Leukemia*. 1997; 11(suppl 1):S7–S11. [PubMed: 9130685]
32. Baylin SB, Herman JG. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. *Trends Genet*. 2000; 16:168–174. [PubMed: 10729832]
33. Robertson KD. DNA methylation, methyltransferase, and cancer. *Oncogene*. 2001; 20:3139–3155. [PubMed: 11420731]
34. Nguyen TT, Mohrbacher AF, Tsai YC, et al. Quantitative measure of c-abl and p15 methylation in chronic myelogenous leukemia: Biological implications. *Blood*. 2000; 95:2990–2992. [PubMed: 10779450]
35. Asimakopoulos FA, Shteper PJ, Krichevsky S, et al. ABL1 methylation is a distinct molecular event associated with clonal evolution of chronic myeloid leukemia. *Blood*. 1999; 94:2452–2460. [PubMed: 10498618]
36. Roman-Gomez J, Castillejo JA, Jimenez A, et al. Cadherin-13, a mediator of calcium-dependent cell-cell adhesion, is silenced by methylation in chronic myeloid leukemia and correlates with pretreatment risk profile and cytogenetic response to interferon alfa. *J Clin Oncol*. 2003; 21:1472–1479. [PubMed: 12697869]
37. Enokida H, Shiina H, Urakami S, et al. Ethnic group-related differences in CpG hypermethylation of the GSTP1 gene promoter among African-American, Caucasian and Asian patients with prostate cancer. *Int J Cancer*. 2005; 116(2):174–81. [PubMed: 15800905]
38. Shen L, Ahuja N, Shen Y, et al. DNA methylation and environmental exposures in human hepatocellular carcinoma. *J Natl Cancer Inst*. 2002; 94:755–761. [PubMed: 12011226]
39. Cameron EE, Bachman KE, Myöhänen S. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet*. 1999; 21:103–107. [PubMed: 9916800]
40. Lugo TG, Pendergast AM, Muller AJ, et al. Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. *Science*. 1990; 247(4946):1079–82. [PubMed: 2408149]
41. Heisterkamp N, Jenster G, Hoeve J, et al. Acute leukaemia in bcr/abl transgenic mice. *Nature*. 1990; 344:251–253. [PubMed: 2179728]
42. Daley GQ, Van Etten RA, Baltimore D. Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. *Science*. 1990; 247:824–830. [PubMed: 2406902]
43. Druker BJ, Guilhot F, O'Brien SG, et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med*. 2006 Dec 7; 355(23):2408–17. [PubMed: 17151364]
44. Cheshier SH, Morrison SJ, Liao X, et al. In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. *Proc Natl Acad Sci USA*. 1999; 96(6):3120–5. [PubMed: 10077647]

Figure 1. *JunB* DNA methylation in CML

(A) Map of the *JUNB* gene. Each vertical line represents a CpG site. The arrow the transcription start site. The vertical lines on top, the restriction sites for *HpyCH4* IV and *Taq* I respectively. The solid black lines below indicate the regions analyzed using bisulfite PCR and RT-PCR assays. (B) MSP analysis of *JUNB* in CML. This was the original assay used by Yang *et al* (19). U: non-methylated reaction; M: methylated reaction. The number on top represents the sample number. SssI the artificially methylated positive control. MW: the molecular weight marker (a 50 bp ladder was used). As shown none of the samples was methylated. (C) COBRA analysis of *JUNB* methylation in CML in region 1 (top) and region 2 (bottom). The number on top indicate the patient sample. SssI the artificially methylated positive control. MW the molecular weight marker. The arrows on the right indicate the restricted (M, methylated) and unrestricted (UM, unmethylated) PCR products. The figure on the right indicated molecular weight.

	clone	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
SssI	7	●	●	●	●	●	●	●	●	●	●	●	○	●	●	○	●	○	○
	3	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	○	●
	9	●	●	●	●	●	○	●	●	●	●	●	●	●	●	●	●	●	●
	6	●	●	●	●	○	●	●	●	●	●	●	●	●	●	●	●	○	●
	8	●	●	●	●	○	●	●	●	●	●	●	●	●	●	●	●	●	●
	1	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
K562	5	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
	4	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	2	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	1	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	3	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	4	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
BP	5	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	1	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	2	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	5	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	6	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	7	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
BP	9	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	8	●	○	●	●	●	○	●	●	●	●	●	●	●	●	●	●	●	●
	4	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	5	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	9	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	7	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
AP	8	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	2	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	6	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	3	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
	9	●	●	○	○	○	○	●	●	●	●	●	○	○	○	●	●	●	○
	7	○	●	●	●	○	○	●	○	●	○	●	●	●	○	●	●	●	○
AP	1	●	●	●	●	●	●	●	●	●	○	●	●	●	●	●	●	○	●
	2	●	●	●	●	●	●	●	●	●	●	●	●	●	●	○	●	●	●
	4	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
	5	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
	8	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●

Figure 2. JUNB bisulfite sequencing of patients and leukemia cell lines

Results of bisulfite sequencing (21) are shown for each CpG site studied (number on top of each column). Black circles methylated CpG site. Open, unmethylated CpG site. SssI, SssI treated positive control; K562, CML cell line; BP, blastic phase; AP, accelerated phase.

Figure on the left, the number of the clone. As shown methylation was observed in most CpG sites of the artificially methylated SssI-treated positive control but rarely in K562 cells or samples with BP CML. AP confirms the methylation status in one of the AP cases.

Figure 3. Analysis of JUNB methylation in Ph-CML

Nine patients with Ph-CML were studied using the MSP assay reported by Yang *et al* (19). As shown in these 5 representative patients, no methylation was observed in any of the patients. Figure on top, patient number. U unmethylated; M, methylated. SssI positive control. MW molecular weight marker. Figure on the right molecular weight.

Figure 4. *JUNB* expression in *BCR-ABL1*-positive cell lines and in patients with CML

(A) Expression of *JUNB* transcripts was analyzed by RT-PCR in healthy volunteers and *BCR-ABL1*-positive cell lines. Markedly low *JUNB* expression was observed in cell lines when compared with normal controls. Data presented as the percentage compared to control.

(B) The expression of *JUNB* was also determined by RT-PCR in 27 patients with CML (10 in CP, 9 in AP, and 8 in BP), as well as in 6 patients with AML and Ph+ ALL and in 5 healthy volunteers and 9 with Ph-CML. No statistically significant differences in *JUNB* level expression were observed between healthy volunteers and patients in CP but progressive decrease expression was observed between CP, AP and BP patients. between normal or CP and advanced phase diseases ($p=0.002$). Levels in Ph-CML and AML were supra-normal compared to normal control and non detectable in Ph+ALL.

Figure 5. Microarray based *JunB* expression analysis in patients with CML

(A) *JUNB* expression in patients with CP-, AP-, and BP-CML was analyzed using microarrays assays. Log ratio was defined as expression compared to that detected in a pool of 200 patients with CP-CML. Positive values denote increased expression while negative values denote gene downregulation decrease repression was observed between CP, AP and BP patients. As shown in BP-CML, there is almost universal downregulation of *JUNB* (right panel figure 4A), whereas expression levels were heterogenous in CP (left panel) and intermediate in AP (middle panel). (B) Median levels of *JUNB* expression as detected by microarray assay. The median decrease in *JUNB* expression in patients with BP-CML samples compared to those obtained from patients with CP-CML was 0.53 logs (ANOVA $p=1.3 \times 10^{-11}$).

Figure 6. *JUNB* expression in patients with CML receiving imatinib

(C) *JUNB* mRNA levels were analyzed sequentially in 9 patients in CP-CML treated with imatinib. As shown, increased *JUNB* levels were observed in 8 of 9 patients analyzed after imatinib therapy. Therapy with the BCR-ABL1 kinase inhibitor imatinib mesylate results in significant expression of *JUNB* transcripts.

Primer characteristics

Table 1

JUNB GeneBank accession number: U20734. Region refers to the area analyzed as shown in figure 1A. MSP (methylation sensitive PCR), M (methylated primers), UM (unmethylated). Primer sequences are shown in a 5' to 3' orientation. F, forward sequence. R, reverse sequence. Primer locations are provided in relation to the translation start site. In parentheses the expected size of the restricted (methylated) fragments.

Region	Assay	Sequence	Restriction enzyme	Primer location	Expected Size (bp)
1	MSP (UM)	F:GACGTTAGGAAAGTTATCGC R:CGAACTAAATACCTAATCGCG	N/A	F: -224 to -205 R: -109 to -89	182
1	MSP (M)	F:TTGGGGGAAATGATGTTAGGAAAGTTATTGT R:ACTACAACAAACAACAAACTCTCCACTACA	N/A	F: -235 to -205 R: -83 to -54	136
1	COBRA	F:AGGGTTTTTGYGTATAGTTGT R:CCCCAACRATCTAAAAAAA	Taq I	F: -169 to 149 R: -28 to -9	161 (108)
2	COBRA	F:GAAATTTTTTATTATGTTTGGGTTTT R:CCTAAACCACACRCCCTTATACC	HpyCH4IV	F: -563 to -535 R: -319 to -297	267 (169)