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Downregulation of *JUNB* mRNA expression in advanced phase chronic myelogenous leukemia

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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.

1. 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, MAFG/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 G_1 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

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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.

2. Materials and methods

2.1. Analysis of DNA methylation and cell lines

DNA methylation was analyzed using three different bisulfite polymerase chain reaction (PCR) assays mapping two different genomic areas in the proximity of the *JUNB* transcription start site (Fig. 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] (Fig. 1A). To further study the promoter region of *JUNB*, we developed a second COBRA assay mapping region 2 (Fig. 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.

2.2. 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.

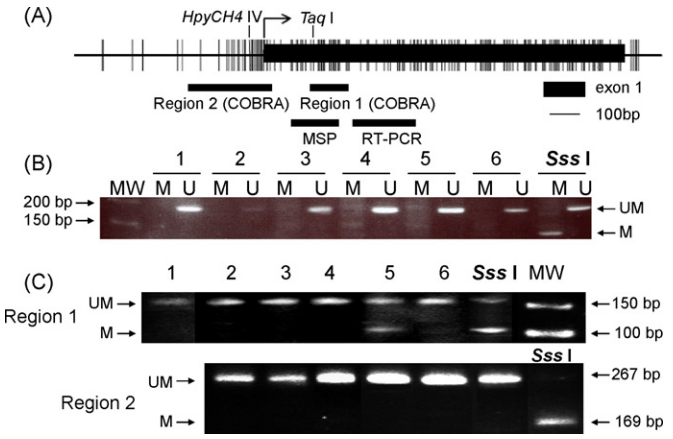

Fig. 1. JunB DNA methylation in CML. (A) Map of the *JUNB* gene. Each vertical line represents a CpG site. The arrow the transcription start site. The vertical lines on top, the restriction sites for *HpyCH4* IV and *Taq* I, respectively. The solid black lines below indicate the regions analyzed using 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.

2.3. 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.

3. Results

3.1. 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 three 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 (Fig. 1B). We then extended this analysis to a total of 102 patients (including the original 62 patients), using the COBRA

Table 1
Primer characteristics.

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:CCCAAACRATCTAAAAAAAA	Taq I	F:−169 to 149 R:−28 to −9	161 (108)
2	COBRA	F:GAAATTTTTTATTTATGTGTTTGGGTTTT R:CCTAAACCACACRCCTTTATACC	HpyCH4IV	F:−563 to −535 R:−319 to −297	267 (169)

JUNB GeneBank accession number: U20734. Region refers to the area analyzed as shown in Fig. 1A. MSP (methylation sensitive PCR), M (methlyated 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.