

## ORIGINAL PAPER

**Promoter hypomethylation of the LINE-1 retrotransposable elements activates sense/antisense transcription and marks the progression of chronic myeloid leukemia**

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Aberrant genome-wide hypomethylation is thought to be related to tumorigenesis by promoting genomic instability. Since DNA methylation is considered an important mechanism for the silencing of retroelements, hypomethylation in human tumors may lead to their reactivation. However, the role of DNA hypomethylation in chronic myeloid leukemia (CML) remains to be elucidated. In this study, the methylation status of the LINE-1 (L1) retrotransposon promoter was analysed in CML samples from the chronic-phase (CP,  $n=140$ ) and the blast crisis (BC,  $n=47$ ). L1 hypomethylation was significantly more frequent in BC (74.5%) than in CP (38%) ( $P<0.0001$ ). Furthermore, L1 hypomethylation led to activation of both *ORF1* sense transcription ( $P<0.0001$ ) and *c-MET* gene antisense transcription ( $P<0.0001$ ), and was significantly associated with high levels of *BCR-ABL* ( $P=0.02$ ) and *DNMT3b4* ( $P=0.001$ ) transcripts. Interestingly, in CP-CML, extensive L1 hypomethylation was associated with poorer prognosis in terms of cytogenetic response to interferon ( $P=0.004$ ) or imatinib ( $P=0.034$ ) and progression-free survival ( $P=0.005$ ). The above results strongly suggest that activation of both sense and antisense transcriptions by aberrant promoter hypomethylation of the L1 elements plays a role in the progression and clinical behavior of the CML.

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**Keywords:** CML; hypomethylation; retrotransposons; LINE-1; blast crisis

**Introduction**

The transition of chronic myeloid leukemia (CML) from the chronic phase (CP) to the blast crisis (BC) is

characterized by the accumulation of molecular and chromosomal abnormalities (Shet *et al.*, 2002), but the molecular mechanisms underlying this genetic instability are poorly understood. A plausible model of disease progression predicts that increased *BCR-ABL* expression promotes the secondary molecular and chromosomal changes essential for the expansion of cell clones with increasingly malignant characteristics, and remains crucial for the malignant phenotype even in advanced stages of the disease (Calabretta and Perrotti, 2004). In fact, an opposite effect of *BCR-ABL* levels on the expression of RAD51, DNA-PKcs, and BRCA1 proteins has been reported, all of them involved in promoting genomic instability associated with defective repair of the DNA double-strand breaks (Deutsch *et al.*, 2001, 2003; Slupianek *et al.* 2001). However, a causal link between decreased repair of DNA double-strand breaks and disease progression, as well as between *BCR-ABL* expression and the secondary genetic changes of BC-CML, has not been yet demonstrated.

Genomic instability is a poorly understood phenotype associated with tumor progression. Several reports have shown that mammalian DNA methylation plays an important role in maintaining genomic stability unrelated to the effects of DNA methylation on gene expression and that hypomethylation of DNA in tumor cells is associated with genomic instability (Chen *et al.*, 1998; Rizwana and Hahn, 1999). Moreover, genomic hypomethylation causes tumorigenesis in mice associated with the acquisition of additional genomic changes (Eden *et al.*, 2003; Gaudet *et al.*, 2003). A link between hypomethylation and the stability of whole chromosome arms is also found in the human Immunodeficiency-Centromeric Instability-Facial Anomalies syndrome (Xu *et al.*, 1999) and human tumors (Lengauer *et al.*, 1997; Wong *et al.*, 2001; Schulz *et al.*, 2002; Eden *et al.*, 2003).

Genome-wide changes in DNA methylation may, in particular, affect those repetitive DNA sequences that are comparatively rich in CpG dinucleotides and

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contain a considerable fraction of total methylcytosine in the genome (Prak and Kazazian, 2000). Retrotransposons are the most important repetitive transposable elements in the human genome. They are copied into RNA, the RNA is reverse-transcribed into DNA, and the DNA is inserted into the genome at a new location (Deininger and Batzer, 2002). The human genome is littered with remnants of the most prominent non-long-terminal repeat (non-LTR) retrotransposon, LINE-1 (L1) element, roughly half a million of which are 5' truncated, inverted, or mutated to inactivity (Ovchinnikov *et al.*, 2002). It also contains roughly 5000 full-length 6 kb L1 elements, 60–100 of which are still capable of retrotransposition (Brouha *et al.*, 2003). The full-length L1 elements contain a 5' untranslated region (5' UTR) with an internal promoter (IP), a 1 kb *ORF1* that encodes a protein with RNA-binding capability, a 4 kb *ORF2* that encodes a protein with endonuclease and reverse transcriptase activities, a short 3' UTR, and a poly(A) tail. The L1 promoter is unusual for two reasons: (i) it contains a CpG island that is very heavily methylated in normal individuals. Methylation includes symmetric and asymmetric methylation at CpG dinucleotides as well as methylation at non-CpG sites (Woodcock *et al.*, 1997). This DNA methylation limits the ability of retroelements to be activated and transcribed and to participate in recombination, and also counteracts their tendency to endanger the stability of the genome by amplification and recombination (Yoder *et al.*, 1997; Yu *et al.*, 2001); (ii) L1s contain within their 5' UTR not only a sense strand promoter for their own transcription, but also an antisense promoter (ASP). This ASP has been shown to provide an alternative transcription start site for a number of human genes including *c-MET*, a receptor tyrosine kinase whose activation by hepatocyte growth factor (HGF) can lead to transformation and tumorigenicity in a variety of tumors (Speek, 2001; Higumann *et al.*, 2002; Birchmeier *et al.*, 2003; Ma *et al.*, 2003). Therefore, it is believed that hypermethylation of L1 is a major defense mechanism to repress these genetic elements that could be otherwise very damaging if actively transcribed.

Hypomethylation-induced retrotransposition of L1 can inactivate tumor suppressor genes (such as *APC* in colon cancer) (Miki *et al.*, 1992) and the L1 promoter inserted in the upstream of the oncogenes (as seen in *c-MYC* in breast cancer) can activate them (Morse *et al.*, 1988). Furthermore, hypomethylation of L1 has been reported in urothelial bladder carcinoma (Florl *et al.*, 1999), malignant testicular tumors (Bratthauer and Fanning, 1992), hepatocellular carcinoma (Lin *et al.*, 2001), chronic lymphocytic leukemia (Dante *et al.*, 1992), and prostate carcinomas (Santourlidis *et al.*, 1999). In addition, in prostatic cancer, L1 hypomethylation was associated with progressive tumors and chromosomal abnormalities (Schulz *et al.*, 2002).

Despite the important role of L1 hypomethylation in promoting genomic instability and cancer progression, its possible relevance in CML has not been evaluated. In the present study, we show that **L1 sense and antisense transcriptions are activated by promoter**

**hypomethylation in CML and that this event is frequently associated with the evolution of the disease to the advanced phase.**

## Results

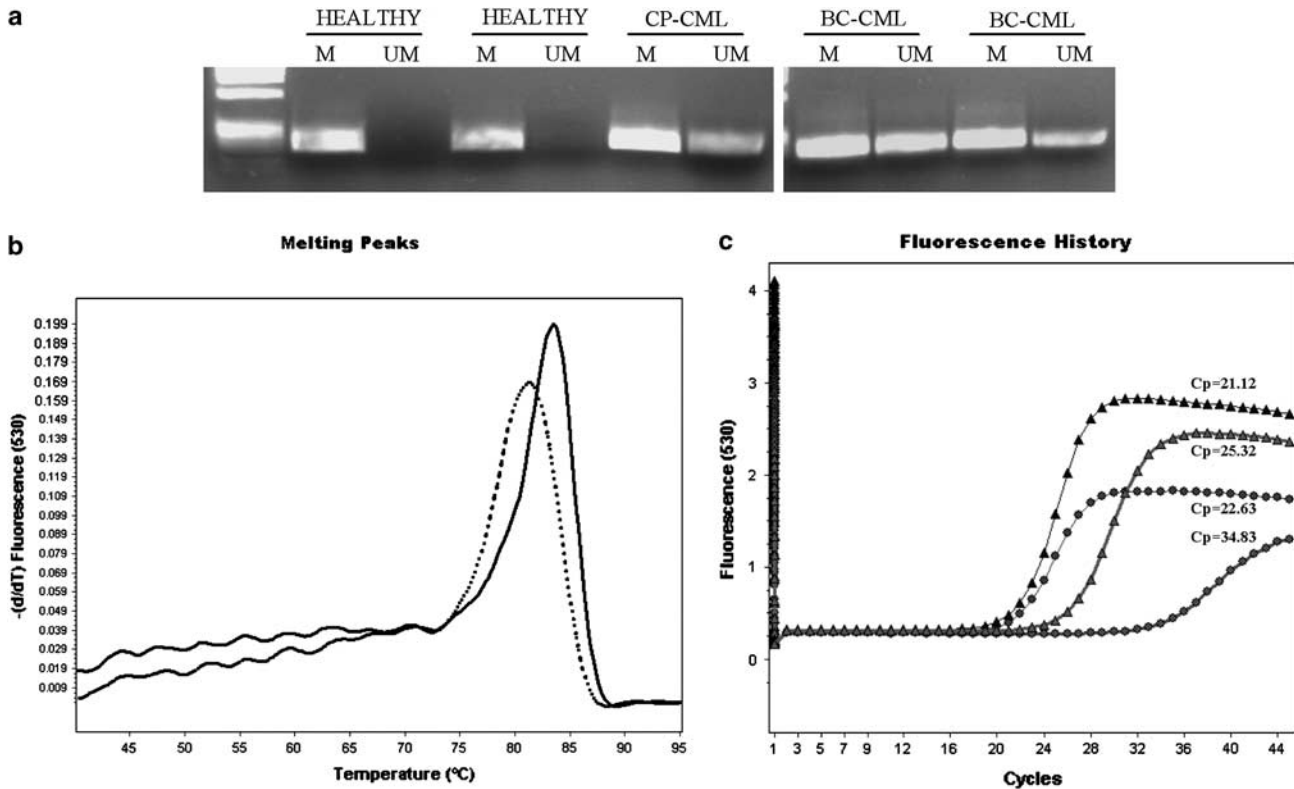
### *L1 is hypermethylated in normal bone marrow cells*

We analysed L1 methylation in 50 normal bone marrow samples. As expected, L1 promoter DNA sequences were strongly methylated in normal bone marrow cells. This is illustrated by the amplification of the methylated sequences with the complete lack of nonmethylated sequence amplification in the majority (36/50, 72%) of these samples (Figure 1a). However, 28% of the non-neoplastic marrow specimens displayed a slight amount of L1 hypomethylation, as defined by the presence of specific products in the melting curves from both methylated (melting temperature 83.4°C) and unmethylated sequences (melting temperature 81.2°C, Figure 1b and c). Based on the background fluorescence intensity, a cutoff level was determined for specific fluorescence for both unmethylated and methylated sequences in normal individuals. This threshold was used to calculate the cycle threshold or crossing point ( $C_P$ ) of each sample. The  $C_P$  value was directly proportional to the amount of target sequence present in the sample. The normal sample, which showed the lowest difference in  $C_P$  between the target (methylated sequences) and the reference (unmethylated sequence) sequences, was used as control/calibrator sample for quantification of the L1 promoter methylation in both healthy individuals and CML patients. It was considered as 100%.

Based on these prerequisites and to determine the cutoff value for altered L1 promoter methylation in CML samples, we quantified by means of qrt-methylation-specific PCR (MSP) the methylation status of the L1 promoter in those healthy donors who showed some degree of unmethylated sequence amplification.  $N_{L1}$  ratios fell between 100 and 231% ( $160 \pm 45.0\%$ ). An  $N_{L1}$  ratio equal to or below 70.0% (determined as the mean minor 2s.d.) was chosen to define hypomethylation of L1 promoter in CML DNA samples.

### *L1 promoter is hypomethylated in CML samples*

By qrt-MSP, CpG island of the L1 promoter was revealed to be highly hypomethylated in Ph-positive CML (K562, KU812, TCC-s, and KYO-1) cell lines (median  $N_{L1}$  ratio: 3%, range: 1–3%), whereas Ph-positive precursor-B (MY, TOM-1, BV173, and NALM-20) cell lines showed normal levels of L1 methylation (median  $N_{L1}$  ratio: 85%, range: 85–90%, Figure 2). Among CML patients, hypomethylation of L1 promoter was more frequently observed in BC (35/47, 74.5%) than in CP (53/140, 38%). This difference was highly significant ( $P < 0.0001$ ). Furthermore, a more profound level of hypomethylation was observed among BC samples compared with CP samples (mean  $N_{L1}$  ratios:  $37.5 \pm 17.1\%$  vs  $46 \pm 18\%$ ,  $P = 0.07$ ).



**Figure 1** Methylation status of L1 promoter in healthy individuals and CML patients. (a) MSP analysis of CpG island within L1 promoter in healthy individuals and CML patients. CP indicates chronic phase CML; BC indicates blast crisis CML; UM, unmethylated sequences; M, methylated sequences. Promoter hypermethylation is observed in normal subjects (no amplification of unmethylated sequences), whereas some degree of L1 hypomethylation was observed among CML patients (amplification of both methylated and unmethylated sequences). (b) qrt-MSP melting curve analysis of a CML patient showing the presence of two specific PCR products: unmethylated (dotted line, melting temperature 81.2°C) and methylated L1 sequences (solid line, melting temperature 83.4°C). (c) L1 promoter methylation level of a representative CML case (lines with triangles) and a control/healthy individual (lines with circles) as measured by qrt-MSP. The difference between the crossing points of the methylated (fine lines) and unmethylated (bold lines) L1 sequences was lower for the CML patient compared with that of the healthy individual, indicating a lower degree of methylation in the CML patient ( $N_{L1}$  ratio for healthy individual: 100%;  $N_{L1}$  ratio for CML patient: 2%.  $N_{L1}$  ratios were calculated as described in Materials and methods)



**Figure 2** Methylation status of L1 promoter in Ph<sup>+</sup>-positive cell lines. MSP analysis of CpG island within L1 promoter in six Ph<sup>+</sup>-positive cell lines. UM, unmethylated sequences; M, methylated sequences. Only K562 and KU812 cell lines showed L1 hypomethylation. A CML sample was used as positive control for methylated and unmethylated reactions

*L1 hypomethylation in CML is associated with activation of ORF1 sense transcription*

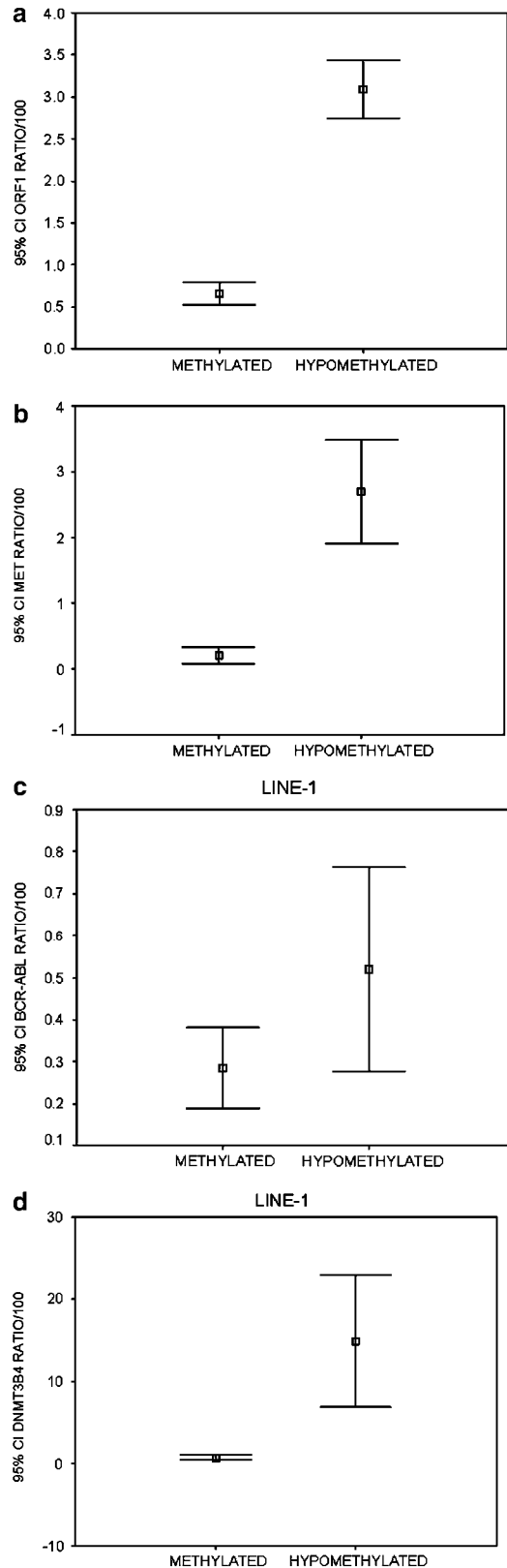
Quantitative expression of *ORF1* transcripts was assessed by means of qrt-PCR using cDNA from a healthy donor as control (it was considered as 100% of  $N_{ORF1}$  ratio). Normalized ratios for *ORF1* were determined in bone marrow specimens from 30 healthy individuals.  $N_{ORF1}$  ratios fell between 37 and 100% (mean  $N_{ORF1}$ :  $68.8 \pm 12.2\%$ ). Among CML patients, those with methylated L1 promoter showed a mean  $N_{ORF1}$  ( $65 \pm 20\%$ ) similar to that found in healthy individuals; however, mean  $N_{ORF1}$  was significantly higher in those CML

patients who showed L1 promoter hypomethylation ( $308.9 \pm 128.1\%$ ,  $P < 0.0001$ , Figure 3a). Moreover, an  $N_{ORF1}$  value equal to or above 130% (determined as the mean  $N_{ORF1}$  from normal individuals plus 5s.d.) was chosen to define overexpression of *ORF1* in CML RNA samples. Using this cutoff value, overexpression of *ORF1* was found in 88% of CML patients with L1 hypomethylated but only in 1% of CML patients with methylated L1 promoter ( $P < 0.0001$ ).

High levels of *ORF1* expression were observed among L1 hypomethylated Ph-positive CML (K562, KU812, TCC-s, and KYO-1) cell lines (mean  $N_{ORF1}$  ratio: 632%, range: 320–1120%), whereas L1 hypermethylated Ph-positive precursor-B (MY, TOM-1, BV173, and NALM-20) cell lines showed normal levels of *ORF1* expression (mean  $N_{L1}$  ratio: 68%, range: 53–92%).

*L1 hypomethylation in CML is associated with activation of c-MET antisense transcription*

L1 has two transcription-regulation regions located in the 5' UTR: an internal or sense promoter driving transcription of the full-length L1, and an ASP driving transcription in the opposite direction into adjacent

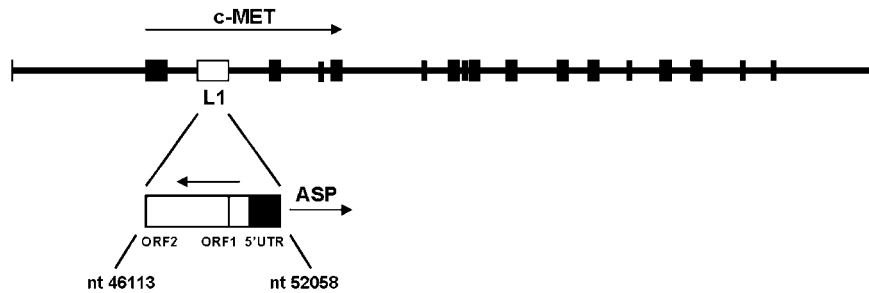


**Figure 3** *ORF1*, *c-MET*, *BCR-ABL*, and *DNMT3b4* expression in CML patients as measured by qrt-PCR. Significantly higher levels of *ORF1* (a), *c-MET* (b), *BCR-ABL* (c), and *DNMT3b4* (d) transcripts were detected among L1 hypomethylated CML patients compared with methylated patients

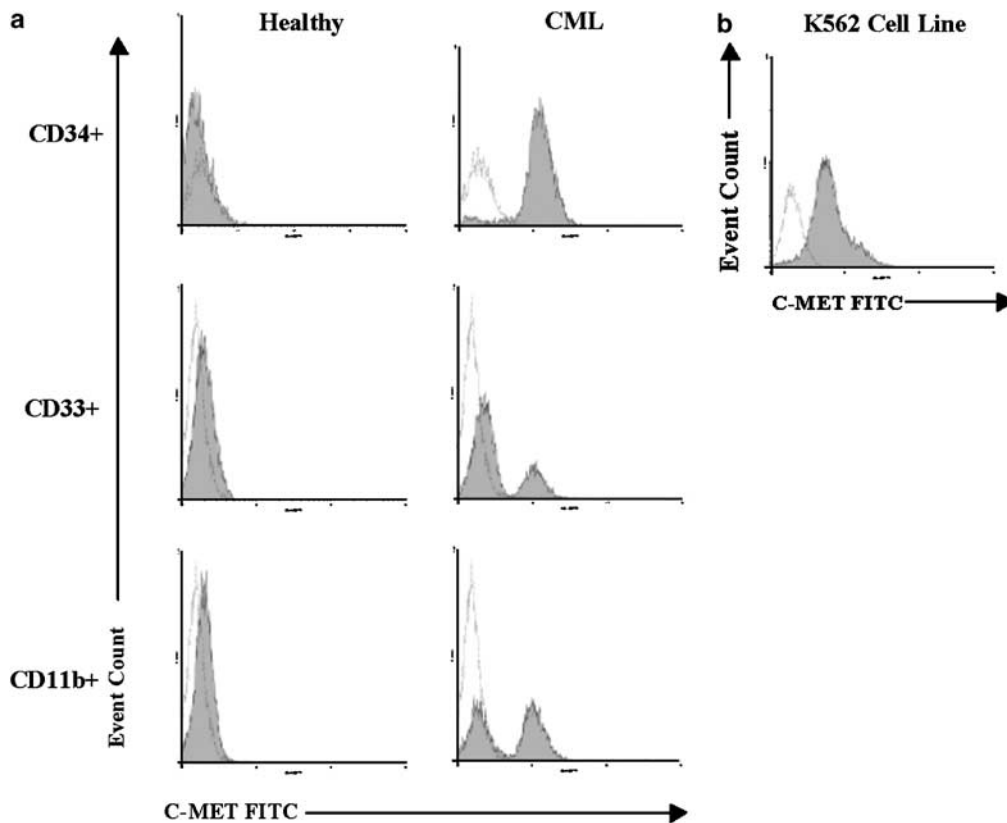
cellular sequences (Speek, 2001; Higumann *et al.*, 2002). Chimeric transcripts derived from the L1 ASP are highly represented in expressed-sequence tag (EST) databases (Speek, 2001; Higumann *et al.*, 2002). One of these ESTs (BF208095, 665 nt, six exons) contains spliced exons identical to those found in the *c-MET* proto-oncogen mRNA, suggesting that L1s containing active ASPs are capable of interfering with normal *c-MET* gene expression (Speek, 2001; Higumann *et al.*, 2002). We performed a search for alignments of the two sequences (full-length L1 and *c-MET* gene) using the BLAST2 finder program (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/b12.html>). Results showed that the full-length L1 sequence was inserted within intron 2 of the *c-MET* gene between 46 113 and 52 058 nt. As shown in Figure 4, transcriptions by the L1 internal/sense promoter and *c-MET* promoter drive in opposite directions. Therefore, *c-MET* gene has the same direction of transcription as the L1 ASP. As transcription of the adjacent genes by L1 ASP is orientation-dependent, which means that only genes having the same transcriptional orientation as the ASP can be transcribed and processed, one could speculate that hypomethylation of L1 promoter might influence *c-MET* expression.

Quantitative expression of *c-MET* transcripts was assessed by means of qrt-PCR using cDNA from a healthy donor as control (it was considered as 100% of  $N_{MET}$  ratio). Normalized ratios for *c-MET* were determined in bone marrow specimens from 30 healthy individuals.  $N_{MET}$  ratios fell between 2 and 100% (mean  $N_{ORF1}$ :  $23.6 \pm 20.2\%$ ). Among CML patients, those with methylated L1 promoter showed a mean  $N_{MET}$  ( $20.6 \pm 35.3\%$ ), similar to that found in healthy individuals; however, mean  $N_{MET}$  was significantly higher in those CML patients who showed L1 promoter hypomethylation ( $269.3 \pm 250.6\%$ ,  $P < 0.0001$ , Figure 3b). Moreover, an  $N_{MET}$  value equal to or above 125% (determined as the mean  $N_{MET}$  from normal individuals plus 5 s.d.) was chosen to define overexpression of *c-MET* in CML RNA samples. Using this cutoff value, overexpression of *c-MET* was found in 61% of CML patients with L1 hypomethylated, but in none of CML patients with methylated L1 promoter ( $P < 0.0001$ ). High levels of *c-MET* expression were observed in two L1 hypomethylated Ph-positive CML cell lines (K562,  $N_{MET}$  ratio: 3267%, and KU812,  $N_{MET}$  ratio: 2834%), whereas Ph-positive CML TCC-s and KYO-1 cell lines and L1 hypermethylated Ph-positive precursor-B (MY, TOM-1, BV173 and NALM-20) cell lines showed normal levels of *c-MET* expression (mean  $N_{L1}$  ratio: 29.8%, range: 1–42%).

In addition, surface expression of c-MET protein was tested in marrow cells from 10 healthy donors. No expression was found in CD34-positive cells, myeloid CD33 cells or CD11b granulocytes. However, L1 hypomethylated CML patients who expressed high levels of c-MET mRNA also displayed c-MET protein expression on CD34 cells, myeloid CD33 cells and granulocytes (Figure 5a). No expression was found on blast cells from five *BCR-ABL*-negative acute leukemias, whereas K562 cell line was positive for c-MET surface expression (Figure 5b).



**Figure 4** Transcriptional regulation of the *c-MET* gene by the L1 ASP. Exons of the *c-MET* gene are represented by black rectangles. Direction of transcription is indicated by arrows. Full-length L1 retrotransposon (white rectangle) is inserted in an inverted position within intron 2 of the *c-MET* gene. Transcriptions by the L1 IP and *c-MET* gene drive in opposite direction. Note that *c-MET* gene has the same direction of transcription as the L1 ASP



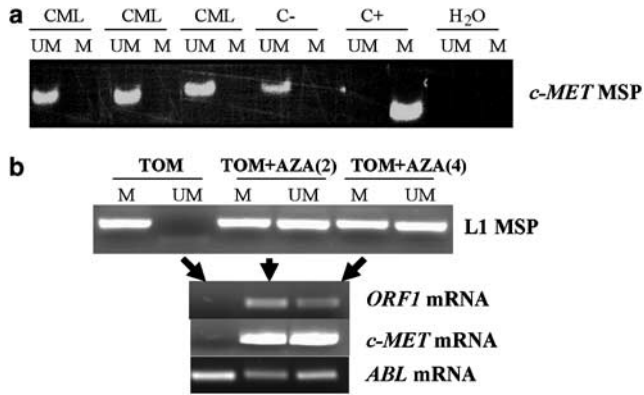
**Figure 5** Expression of c-MET protein on normal and CML marrow cells. The expression of surface c-MET protein on normal and CML marrow cells was measured by multiparametric flow cytometry using anti-c-MET MnAb. Histograms are shown after gating on viable cells (IP low vs SSC) and specific cell subsets (CD34, CD33 or CD11b-PE vs SSC). Shaded-solid lines represent c-MET expression and blank-dotted lines FITC-conjugated isotype control antibody. No expression was observed in healthy individuals, whereas L1 hypomethylated CML patients showed c-MET expression in all the cell subsets studied (a). K562 cell line which expressed c-MET protein was used as positive control (b)

The possibility that the high levels of *c-MET* transcripts detected in CML were determined by hypomethylation of its own promoter was ruled out by means of MSP. Promoter of *c-MET* gene was invariably unmethylated in all CML cases and healthy individuals, suggesting that changes in the levels of *c-MET* mRNA do not depend on the epigenetic regulation of its own promoter (Figure 6a). Moreover, to determine the relationship between L1 hypomethylation and *ORF1/c-MET* expression, we treated the Ph<sup>+</sup>-positive ALL cell lines TOM-1 and NALM-20 (both with L1 methylated

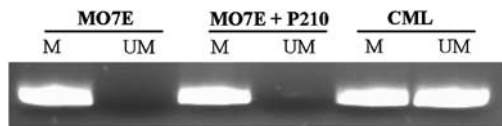
and nonexpressing *ORF1* or *c-MET*) with the demethylating agent 5'-aza-2'-deoxycytidine (AZA). This treatment induced L1 hypomethylation and expression of both *ORF1* and *c-MET* transcripts (Figure 6b).

*L1 hypomethylation in CML is associated with BCR-ABL and DNMT3b4 transcript levels*

Several lines of evidence demonstrate that the mere presence of the Ph-chromosome is not sufficient to produce hypomethylation of the L1 promoter: (1) L1 is



**Figure 6** Effect of L1 hypomethylation on sense/antisense transcription. (a) MSP analysis of CpG island within *c-MET* gene promoter in a healthy individual (C-) and CML patients. Lack of promoter methylation at *c-MET* gene is observed in all the cases. C+ indicates positive control for methylated reactions (human genomic DNA universally methylated). (b) Effects of AZA treatment on L1 sense/antisense transcription in the TOM-1 Ph<sup>+</sup>-positive precursor-B ALL cell line (L1 hypermethylated and lacking *ORF1/c-MET* expression). Cells were treated with 2 or 4  $\mu$ M of AZA for 4 days. AZA treatment induced hypomethylation of the L1 promoter and expression of *ORF1* and *c-MET* transcripts



**Figure 7** Effect of p210<sup>BCR-ABL</sup> transfection on L1 promoter methylation. Transfection of the MO7e Ph<sup>-</sup>-negative myeloid cell line (L1 hypermethylated) with p210<sup>BCR-ABL</sup> did not produce changes in the methylation status of the L1 promoter. CML patient was used as positive control for unmethylated and methylated reactions

normally methylated in a group of CML patients; (2) L1 hypomethylation was not observed in Ph<sup>+</sup>-positive ALL cell lines, and (3) transfection of the L1 hypermethylated MO7e cell line with p210<sup>BCR-ABL</sup> was not associated with changes in the L1 methylation status ( $N_{L1}$  ratio of MO7e: 91%;  $N_{L1}$  ratio of p210-MO7e: 89%, Figure 7). However, L1 hypomethylation was associated with high levels of *BCR-ABL* expression. Hypomethylated patients showed higher *BCR-ABL* transcript levels (mean  $N_{BCR-ABL}$  ratio:  $52 \pm 44\%$ ) than patients with methylated L1 (mean  $N_{BCR-ABL}$  ratio:  $28.3 \pm 26.7\%$ ,  $P=0.02$ , Figure 3c). Furthermore, L1 hypomethylated patients had higher levels of *DNMT3b4* expression (mean  $N_{DNMT3b4}$  ratio:  $1400 \pm 1502.7\%$ ) than L1 methylated patients (mean  $N_{DNMT3b4}$  ratio:  $73.3 \pm 39.6\%$ ,  $P=0.001$ , Figure 3d).

#### L1 hypomethylation, response to treatment, and clinical outcome

The clinical and laboratory characteristics of CP-CML patients with hypomethylated and normal L1 at diagnosis are shown in Table 1. Individual factors such as sex, age, spleen size, percentage of blast cells in the peripheral blood, platelet count, hemoglobin level, and

**Table 1** Clinical characteristics at diagnosis and outcome of 140 chronic myeloid leukemia patients according to L1 methylation status

Feature	L1 hypomethylated (n = 53)	L1 methylated (n = 87)	P
Sex (M/F), %	55/45	56/44	NS
Age, median (int. r)	48 (35–59)	49 (37–57)	NS
Palpable spleen, %	62	56	NS
Median hemoglobin (g/l) (int. r)	120 (97.7–129.3)	110 (83.5–119.1)	NS
WBC $\times 10^9/l$ , median (int. r)	163 (61.2–348.1)	141 (53.1–228.5)	NS
Median platelet count $10^9/l$ (int. r)	375 (281–652)	401 (264–612)	NS
Median peripheral blood blast as % WBC (int. r)	1 (0–4.1)	1 (0–3.6)	NS
Sokal score, %			
High	34	25	NS
Low/Intermediate	66	75	
Kantarjian score, %			
Stage 3	48	42	NS
Stages 1–2	52	58	
Hasford score, %			
High	19	25	NS
Low/Intermediate	81	75	
Treatment type, %			
Chemotherapy	27	29	NS
Interferon	54	54	
Imatinib	19	17	
Transplantation	28	25	
Response to treatment, %			
CHR	78	92	0.05
MCR with IFN	3	32	0.004
MCR with imatinib	47	100	0.034
Disease progression	63	24	0.03
Death	45	31	0.08

(int. r) indicates interquartile range; CHR, complete hematologic response; MCR, major cytogenetic response; IFN, interferon

WBC count were not significantly associated with L1 methylation status. When L1 methylation status was correlated with pretreatment risk groups, no significant association between L1 hypomethylation and high-risk patients assessed by the Sokal, Hasford, and Kantarjian scoring systems was found.

CML patients in this study were treated with chemotherapy (mainly, hydroxyurea,  $n=39$ ), IFN-based regimens ( $n=76$ ), or imatinib ( $n=25$ ). In all, 37 patients received stem cell transplantation (five autologous, 32 allogeneic). The treatment modality and number of patients who received transplantation were similarly distributed between the two L1 methylation groups (Table 1). Moreover, the mean times of IFN administration were similar in methylated (30 months) and hypomethylated (30.2 months) patients. Hypomethylation of L1 promoter was correlated with a poor response to treatment (Table 1). Thus, the complete hematologic response (CHR) rate was significantly lower among hypomethylated patients than in patients with methylated L1 promoter (78 vs 92%,  $P=0.05$ ).

Among 76 patients under IFN therapy, a ‘major’ cytogenetic response was observed in 32% of patients with methylated L1, but only in 3% of hypomethylated patients ( $P=0.004$ ). A multivariate analysis including the clinical factors described in Table 1 demonstrated the methylation status of the L1 promoter to be the only independent factor predicting the cytogenetic response to IFN (Table 2). Among the 25 CP-CML patients receiving imatinib as front-line therapy, the rate of major cytogenetic response at 6 months was 100% in the L1 methylated group, as compared with 47% in the L1 hypomethylated group ( $P=0.034$ ). In addition, the molecular response rate (reduction of the  $N_{BCR-ABL}$  ratio greater than 2 log) was better for patients with methylated L1 (80%) as compared with L1 hypomethylated patients (18%,  $P=0.036$ ).

Survival data were available from all patients. During the study period, hypomethylated patients had higher progression (63 vs 43%,  $P=0.03$ ) and mortality rate (45 vs 31%,  $P=0.08$ ) than patients with methylated L1 (Table 1). Kaplan–Meier analysis revealed differences in the length of the CP (Figure 8). The estimated median PFS time for hypomethylated patients was 58.3 months (95% confidence interval (CI), 33.1–83.5) compared to 96.8 months (95% CI, 82.5–111.1) for patients with methylated L1 ( $P=0.005$ ). Multivariate analysis using a forward stepping model showed that the methylation

status of the L1 promoter ( $P=0.02$ ) and the Hasford risk group ( $P=0.04$ ) were the only independent factors associated with PFS (Table 3).

## Discussion

From three lines of evidence, the present study indicates that L1 promoter hypomethylation is an important feature in CML. Firstly, hypomethylation increased from non-neoplastic marrow cells toward CML cells. Secondly, an increase in L1 hypomethylation in the CML patients with advanced disease was observed. To our knowledge, the hypomethylation of L1 promoter is the most common molecular abnormality associated with BC-CML reported to date. Thirdly, high levels of hypomethylation were an independent marker of poor prognosis in a subset of patients with CP-CML.

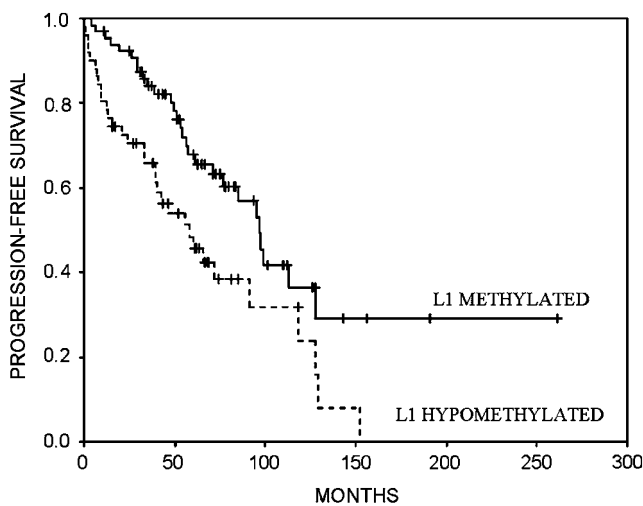
Two mechanisms are possible to explain how L1 hypomethylation could influence CML:

(a) A legitimate model of disease progression in CML predicts that increased  $BCR-ABL$  activity promotes the accumulation of molecular and chromosomal alterations directly or indirectly responsible for the malignant phenotype of BC-CML cells (Calabretta and Perrotti, 2004). The significant association between high levels of  $BCR-ABL$  expression and L1 hypomethylation observed in our study suggests that L1 hypomethylation could be one of such mechanisms employed by  $BCR-ABL$  to generate an unstable genome in the malignant cell. Dereglulation of DNA methylation is thought to contribute to genomic instability in cancer. Reactivation of retroelements might represent a particularly important consequence of DNA hypomethylation, since their containment is a key function of DNA methylation in mammalian genomes (Yoder *et al.*, 1997). Retrotransposition of L1 sequences results in a highly unstable branched DNA structure prone to undergoing recombination with accessible elements located nearby or even elsewhere in the genome (Feng *et al.*, 1996). Chromosome deletions and translocations probably caused by retrotransposition events have indeed been observed in several human cancers (Morse *et al.*, 1988; Nagarajan *et al.*, 1990; Miki *et al.*, 1992; Pomykala *et al.*, 1994; Liu *et al.*, 1997). Moreover, a significant increase in the expression of a variety of retrotransposons and direct evidence for the hypomethylation of L1s has been observed during human cellular transformation (Takai *et al.*, 2000; Menendez *et al.*, 2004; Suter *et al.*, 2004).

**Table 2** Multivariate regression analysis for response to interferon treatment in 76 CML patients

Variable	Level of significance	
	P	P*
L1 hypomethylation	0.001	—
Kantarjian score	0.9	0.1
Sokal score	0.9	0.2
Hasford score	0.1	0.2

\*Significance after adjustment for L1 methylation status



**Figure 8** Kaplan–Meier survivor function for CML patients. PFS curve for all the patients enrolled in this study according to the L1 methylation status

**Table 3** Multivariate Cox model for progression-free survival in 140 CML patients

Variable	Level of significance	
	P	P*
L1 hypomethylation	0.02	—
Kantarjian score	0.1	0.2
Sokal score	0.3	0.4
Hasford score	0.03	0.04

\*Significance after adjustment for L1 methylation status



It is evident that L1 hypomethylation occurs in many BC-CML patients, but whether this epigenetic change is a cause or consequence of progression seems to be unclear. Our data argue against the second alternative because L1 hypomethylation was not only associated with BC but also with a cohort of CP-CML patients. Interestingly, these patients progress to BC much more rapidly than CP patients lacking hypomethylation, suggesting that hypomethylated patients *ab initio* may be more prone to genomic instability, resembling those patients with deletions of the derivative chromosome 9 (Huntly *et al.*, 2003).

(b) The 5' UTR of L1 houses not only an IP but also an ASP that drives transcription of adjacent cellular genes (Speck, 2001; Higumann *et al.*, 2002). L1 insertions are frequently found in A+T-rich DNA. As coding sequences tend to be more G+C-rich, L1 elements, by preferring A+T-rich DNA, may be more successful genomic parasites, because they can reach higher copy numbers without causing too many deleterious insertions. However, L1s can and do insert into genes (Kazazian and Moran, 1998). We have found that one of these genes is the *c-MET* proto-oncogene. In fact, the *c-MET* gene has the same direction of transcription as the L1 ASP, which is inserted in the *c-MET* intron 2. Our paper demonstrates that epigenetic changes in the L1 promoter alter the expression of the *c-MET* gene. Hypomethylation of the L1 promoter was associated with overexpression of *c-MET* mRNA and the presence of *c-MET* protein in the CD34+ precursor cell compartment of CML patients.

*c-MET*, which has been found to be overexpressed in a variety of human tumors, is a unique receptor tyrosine kinase with versatile role in regulating numerous biological functions in response to HGF (Comoglio, 2001; Maulik *et al.*, 2002; Birchmeier *et al.*, 2003; Ma *et al.*, 2003). As *c-MET* is overexpressed in CML patients with advanced disease or poor response to conventional treatment, we can speculate that the biological abnormalities in CML cells from these groups of patients depend on the combined action of both tyrosine kinases, BCR-ABL and *c-MET*. Interestingly, whereas imatinib mesylate inhibits the BCR-ABL tyrosine kinase, it has no inhibitory effect on *c-MET* (Buchdunger *et al.*, 2000). Therefore, these findings provide a basis for using HGF/*c-MET* signaling inhibitors as part of the therapeutic approach for poor-risk CML patients (Christensen *et al.*, 2003).

Although genome-wide hypomethylation is generally observed in cancers, its mechanism remains unclear. As maintenance of the fidelity of CpG dinucleotide methylation through DNA replication requires a tightly regulated DNMT expression in replicating cells, deregulation of DNMTs might lead to hypomethylation. It is well established that CML cells show phase-dependent expression of DNMTs (Mizuno *et al.*, 2001). In the CP, levels of DNMTs are not significantly different from those in normal cells. However, cells in the acute phase show an increase in the levels of DNMT1, 3a, and 3b. This suggests that upregulated DNMTs may contribute to the pathogenesis of BC by inducing DNA hyper-

methylation. This hypothesis seems to be in disagreement with our finding concerning frequent L1 hypomethylation in BC-CML. In order to clarify this issue, we have studied the expression levels of *DNMT3b4*, a splice variant of *DNMT3b* whose overexpression correlates significantly with DNA hypomethylation in the pericentromeric satellite region because it lacks the conserved methyltransferase motifs IX and X and, therefore, it does not show DNMT activity (Saito *et al.*, 2002). Our results demonstrate that overexpression of *DNMT3b4* was significantly correlated with the degree of L1 hypomethylation, suggesting that *DNMT3b4* may compete with other DNMT variants for targeting to L1 promoter in CML patients.

In conclusion, our results strongly suggest that activation of both sense and antisense transcriptions by aberrant promoter hypomethylation of the L1 elements plays a role in the pathogenesis of the evolution of CML to its advanced phase, as well as in the clinical behavior of the disease in CP.

## Materials and methods

### Cell lines and samples

Four human Ph'-positive CML (K562, KU812, KYO-1, and TCC-S) and four human Ph'-positive precursor-B ALL (BV173, TOM-1, MY, and NALM-20) cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The megacaryoblastic MO7e cell line and its human p210<sup>BCR-ABL</sup>-overexpressing derivative were kindly provided by Dr JE Dick (University of Toronto, Canada). The cells were cultured in the appropriate medium until harvested for extraction of DNA and RNA. Heparinized bone marrow cells were collected from patients with CML, and from healthy marrow donors. Immediately after harvest, total WBCs were obtained by dextran sedimentation or by red cell lysis of centrifuged buffy coat preparations. Mononuclear cells (MNCs) were isolated from BC-CML and donors by sedimentation on Ficoll-Hypaque gradients. More than 90% of the MNC populations from acute-phase CML were leukemic blasts. We studied 140 patients with Ph-positive CP-CML, diagnosed between August 1982 and December 2003. The study was approved by the Investigational Review Board in accordance with the policies of the Department of Health and Human Services. All patients gave informed consent for the use of their samples. The patients were unselected for type of front-line therapy (39 patients received chemotherapy alone, 76 interferon (IFN) plus citarabine, and 25 imatinib). A diagnostic sample in CP was available for analysis in all patients. Paired samples, where both a diagnostic sample and a BC sample were available, were analysed in 47 patients (34 in myeloid BC and 13 in lymphoid BC). BC was defined by the presence of at least 30% blasts in the blood or bone marrow or extramedullary involvement. Risk categories according to the Sokal, Kantarjian, and Hasford score systems were determined as described previously (Sokal *et al.*, 1984; Hasford *et al.*, 1998; Kantarjian *et al.*, 1999). Hematologic and cytogenetic responses to IFN and imatinib treatments were evaluated. CHR was defined by a WBC count of less than 10 000 mm<sup>3</sup>, a platelet count of less than 450 000 mm<sup>3</sup>, the presence of less than 5% myelocytes plus metamyelocytes, the presence of less than 20% basophils and the absence of blasts and promyelocytes in peripheral blood, and the absence of extramedullary involvement.



Cytogenetic response was assessed on the basis of G-banding in at least 20 cells in metaphase per sample and was defined as ‘major’, comprising complete responders (CR, 0% Ph+ metaphases) and partial responders (PR, 1–34% Ph+ metaphases), or ‘poor’, including patients with a minor (MinR, 35–94% Ph+ metaphases), or no response (NR, 95–100% Ph+ metaphases).

*Semiquantitative real-time methylation specific PCR (qrt-MSP) of L1 promoter*

Analysis of the L1 promoter (GeneBank: X58075, LINE-1 promoter consensus sequence) has revealed that L1 promoter possesses a 372 bp CpG island located between 49 and 420 nt, showing >60% C + G content and an observed-overexpected CpG frequency of >0.6. Promoter methylation of L1 retro-transposon was determined by the MSP method as reported previously (Hermann et al., 1996). MSP distinguishes unmethylated alleles of a given gene based on DNA sequence alterations after bisulfite treatment of DNA, which converts unmethylated but not methylated cytosines to uracils. Subsequent PCR using primers specific to sequences corresponding to either methylated or unmethylated DNA sequences was then performed. Briefly, 1 µg of genomic DNA was denatured by treatment with NaOH and modified by sodium bisulfite. DNA samples were purified using Wizard DNA purification resin (Promega Corp., Madison, WI, USA), treated with NaOH, precipitated with ethanol, and resuspended in 20 µl of water. Qrt-MSP was performed in a rapid fluorescence thermal cycler with three-colour fluorescence monitoring capability (LightCycler, Roche), using 1 µl of bisulfite-modified DNA in 10 µl reaction volume with 0.4 µmol/l each primer, and 1 µl of 10 × LightCycler FastStar DNA Master SYBR Green I (Roche Molecular Biochemicals). The final Mg<sup>2+</sup> concentration in the reaction mixture was adjusted to 3.5 mmol/l. Amplification of the L1 methylated sequences was used as target gene (forward, 5'-GTCGAATAGGAATAGTTTTCGG-3'; reverse, 5'-ACTCCCTAACCCCTTACGCT-3'). The following program conditions were applied for qrt-MSP running: denaturation program, consisting in one cycle at 95°C for 10 min; amplification program, consisting in 45 cycles at 95°C for 10 s, 65°C for 10 s, and 72°C for 10 s; melting program, one cycle at 95°C for 0 s, 40°C for 60 s, and 90°C for 0 s; and cooling program, one cycle at 40°C for 60 s. The temperature transition rate was 20°C/s, except in the melting program, which was 0.4°C/s between 40 and 90°C. Amplification of the L1 unmethylated sequences for each sample was performed as reference gene (forward, 5'-GTTGAATAGGAATAGTTTTGGTTT-3'; reverse, 5'-ACTCCCTAACCCCTTACACTT-3'). It was amplified in the same run and following the same procedure described above for methylated sequences. A procedure based on the relative quantification of target sequence (methylated sequences) vs their controls/calibrators in relation to the reference sequence (unmethylated sequences) was used to assess the degree of L1 promoter methylation. Calculations were automatically performed by LightCycler software (RealQuant, version 1.0, Roche). The normalized ratio was obtained from the following equation and expressed as percentage of the control/calibrator:

$$\text{Normalized ratio } (N_{L1}) = \frac{(E_{\text{target}})^{\Delta C_{\text{p target}}(\text{control-sample})}}{(E_{\text{ref}})^{\Delta C_{\text{p ref}}(\text{control-sample})}}$$

Efficiencies (E) of each gene were calculated from the slopes of crossover points (Cp) vs DNA concentration plot, according to the formula  $E = 10^{(-1/\text{slope})}$ . ΔCp corresponded to the

difference between control/calibrator Cp and sample Cp, either for the target or for the reference sequences. The selected control/calibrator was the bone marrow specimen from a healthy donor. It was considered as 100% (this is not an absolute value indicating a fully methylated patient, but a relative value used as a measure for the relative level of L1 methylation in the particular sample). Water blanks were included with each assay. Results were confirmed by repeating bisulfite treatment and MSP assays for all samples. Occasionally, equal amounts of PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

*Expression of ORF1, c-MET, BCR-ABL, and DNA methyltransferase 3b4 (DNMT3b4) transcripts*

Expression of *ORF1* (GeneBank: M19503 and M59450, Human LINE-1 repeat mRNA with two open reading frames), *c-MET* (GeneBank: X54559, *Homo sapiens* mRNA for *MET* proto-oncogene), and *DNMT3b4* (GeneBank: AF129268, *Homo sapiens DNMT3b4* mRNA) transcripts were analysed by the RT-PCR technique. Total RNA was extracted from marrow samples with Ultraspec (Biotecx, Houston, TX, USA) following the manufacturer's instructions. Reverse transcription was performed on 1 µg total RNA, after heating at 70°C for 5 min, with random hexamers as reaction primer. The reaction was carried out at 42°C for 45 min in the presence of 12U Avian Myeloblastosis virus reverse transcriptase (Boehringer-Mannheim, Germany). Quantitative real-time PCR (qrt-PCR) for *ORF1*, *c-MET* and *DNMT3b4* expression was performed with the LightCycler technology, using 1 µl of cDNA in 20 µl reaction volume with 0.4 µmol/l each primer (*ORF1*-forward: 5'-CCCCAATCTAGCAAGG-3'; *ORF1*-reverse: 5'-AGAGATCCGCTGTAGT-3'; *c-MET*-forward: 5'-GGTCAATTCAGCGAAGTCCT-3'; *c-MET*-reverse: 5'-CCAGTGTGTAGCCATTTTGG-3'; *DNMT3b4*-forward: 5'-CGGGATGAACAGTTAAAGAAAGTA-3'; *DNMT3b4*-reverse: 5'-CCAAAGATCCTTTTCGAGCTC-3'), and 2 µl of 10 × LightCycler FastStar DNA Master SYBR Green I (Roche Molecular Biochemicals). The final Mg<sup>2+</sup> concentration in the reaction mixture was adjusted to 3.5 mmol/l. The following program conditions were applied for qrt-PCR running: denaturation program, consisting in one cycle at 95°C for 8 min; amplification program, consisting in 45 cycles at 95°C for 5 s, 60°C for 10 s and 72°C for 15 s; melting program, one cycle at 95°C for 0 s, 40°C for 60 s and 90°C for 0 s; and cooling program, one cycle at 40°C for 60 s. The temperature transition rate was 20°C/s, except in the melting program, which was 0.4°C/s between 40 and 90°C. *BCR-ABL* expression was detected as reported previously (Emig et al., 1999). *Abelson* gene (*ABL1*) was employed as reference gene, and it was amplified in the same run and following the same procedure described above (forward: 5'-CCCAACCTTTTC GTTGCAGTGT-3'; reverse: 5'-CGGCTCTCGGAGGAGAC GTAGA-3'). In order to reduce the variation between different assays and samples, a procedure based on the relative quantification of target genes vs their controls/calibrators in relation to the reference gene was used. Calculations were automatically performed by LightCycler software (RealQuant, version 1.0, Roche). The normalized ratios ( $N_{\text{ORF1}}$ ,  $N_{\text{MET}}$ ;  $N_{\text{BCR-ABL}}$  and  $N_{\text{DNMT3b4}}$ ), expressed as percentage of the control/calibrator, were obtained by RealQuant software as described above for qrt-MSP of L1 promoter. The selected controls/calibrators were the Ph<sup>+</sup>-positive K562 cell line for *BCR-ABL* transcripts and bone marrow specimens from healthy donors for *ORF1*, *c-MET*, and *DNMT3b4* transcripts. They were considered as 100% expression.

*MSP and unmethylated-specific PCR assay of the c-MET promoter and 5' UTR*

In order to determine the methylation status of the CpG dinucleotides located in the promoter and 5' UTR of the *c-MET* gene, bisulfite-modified DNA was analysed by means of MSP. A pair of primers, MF 5'-GATATTCGTTTTTAA GCGTTAG-3' and MR 5'-ACTCCCTAACCCCTACGCT-3', was designed for the methylated sequence of *c-MET* promoter and UTR. A pair of primers, UF 5'-TGGGTGGGGTAGA GGTGG-3' and UR 5'-ATCCATCCCTAATCCACAAA-3', was designed for the unmethylated sequence of the same region also. Initial denaturation at 94°C for 5 min was followed by 35 cycles of a denaturation step at 94°C for 1 min, an annealing step at 60°C for 1 min, and an extension step at 72°C for 1 min, and a final extension step of 72°C for 7 min was added. The products were separated by electrophoresis on 2% agarose gel. Human male genomic DNA universally methylated for all genes (Intergen Company, Purchase, NY, USA) was used as a positive control for methylated alleles. Water blanks were included with each assay. PCR products were visualized on 2% agarose gels stained with ethidium bromide. Results were confirmed by repeating bisulfite treatment and MSP assays for all samples.

*Detection of c-MET protein by flow-cytometric analysis*

Total marrow cells ( $1-5 \times 10^5$ ) were incubated with unconjugated mouse anti-human c-MET monoclonal antibody (MoAb) to detect the extracellular epitope of human MET protein (Clone DO-24, Upstate Biotechnology, Lake Placid, NY, USA). After two washes with phosphate-buffered saline containing 0.2% bovine serum albumin and 0.2% sodium azide (PBSA), cells were incubated with FITC-labelled goat anti-mouse MoAb (Dako, Denmark). Isotype-matched control IgG<sub>2k</sub> was used to set up regions. PE-conjugated MoAbs against human CD34, CD33, CD11b, CD19, and CD3 were used to identify stem cells, myeloid cells, granulocytes, B-lymphocytes, and T-lymphocytes, respectively. Erythrocytes were lysed after staining by adding 2.5 ml of lysis solution (0.155 mol/l NH<sub>4</sub>Cl + 0.01 mol/l KHCO<sub>3</sub> + 10<sup>-4</sup> mol/l EDTA). Finally, nonviable cells were excluded using propidium iodide (Sigma, St Louis, MO, USA) at 1 µg/ml. Three-color acquisition and analysis were performed using a dual laser FACScalibur™ flow cytometer with Cell Quest Software.

*AZA treatment*

ALL-derived TOM-1 and NALM-20 cell lines were grown at a density of 750 000 cells/ml in 25 cm<sup>2</sup> flasks with 8 ml of RPMI

1640 medium supplemented with 20% fetal bovine serum and maintained at 37°C in a humid atmosphere containing 5% CO<sub>2</sub>. Each of the cell lines was treated with 2 and 4 µM of AZA (Sigma-Aldrich, Steinheim, Germany) for 4 days. After treatment, cells were washed in PBS, pelleted by centrifugation at 1500 r.p.m. during 5 min and used for genomic DNA and RNA isolation. DNA was extracted using QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) and total RNA using RNeasy® Mini Kit (Qiagen, Hilden, Germany). In all, 1 µg of total RNA was used for cDNA synthesis using SuperScript™ II RNase H-RT (Invitrogen Life Technologies, Paisley, UK) with random hexamers.

*Statistical analysis*

All calculations were performed with the SPSS statistical package (SPSS, Chicago, IL, USA). Medians, s.d. and interquartile ranges were calculated for age and clinical and laboratory findings at diagnosis for patients with and without L1 hypomethylation and tested for any significant differences with the Mann-Whitney *U*-test (for continuous variables) or  $\chi^2$  analysis and Fisher exact test (for categorical variables). Overall survival (OS) was calculated from time of diagnosis to death from any cause and was censored only for patients known to be alive at last contact. Progression-free survival (PFS) was measured from CML diagnosis to the appearance of BC or death without disease progression, and it was censored only for those patients alive and without evidence of progression at last follow-up. For both OS and PFS calculations, bone marrow transplant recipients were censored at the time of transplantation. Distributions of OS and PFS curves were estimated by the method of Kaplan and Meier, with 95% confidence intervals calculated by means of Greenwood's formula. Comparisons of OS and PFS between groups were based on the log-rank test. Comparisons adjusted for significant prognostic factors were based on Cox regression models and hazard regression models. All progression and survival data were updated on May 31, 2004, and all follow-up data were censored at that point.

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