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Brief communication

# Repetitive DNA hypomethylation in the advanced phase of chronic myeloid leukemia

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**Abstract**

Repetitive elements are heavily methylated in normal tissues, but hypomethylated in malignant tissues, driving the global genomic hypomethylation found in cancer. This hypomethylation results in chromosomal instability, a well-characterized feature of the advanced phases of chronic myeloid leukemia (CML). We investigated methylation changes of DNA repetitive elements (LINE1, Alu, Satellite-alpha and Satellite-2) during the progression of CML from chronic phase (CP) to blast crisis (BC). CP-CML samples were significantly more hypomethylated for all repetitive sequences compared with normal samples. Furthermore, a more profound level of hypomethylation was observed among BC samples compared with CP samples. Our data suggest that repetitive DNA hypomethylation are closely associated with CML progression.


*Keywords:* LINE1; Alu; Satellite-alpha; Satellite-2; Hypomethylation; CML

## 1. Introduction

Changes in human DNA methylation patterns are an important feature of cancer development and progression. The cancer genome is frequently characterized by promoter hypermethylation of specific genes concurrently with an overall decrease in the level of five methylcytosine [1]. Since interspersed repetitive elements (LINE1 retrotransposons and Alu sequences) as well as tandem repeated centromeric and juxtacentromeric repeats (satellite sequences) contain numerous CpG dinucleotides, the methylation status of these sequences is relevant to understanding global DNA methylation. It is generally thought that repetitive elements are heavily methylated in normal somatic tissues, but are methylated to a lesser extent in malignant tissues, driving the global genomic hypomethylation commonly found in human cancers [2]. This hypomethylation affecting repeat sequences and transposable elements is believed to result in chromosomal instability and increased mutation events [3].

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 [4]. Although, the molecular mechanisms underlying this genetic instability are poorly understood, epigenetic changes seem to play an important role. In fact, we have recently shown that the sense and antisense transcriptions of the LINE1 (L1) retrotransposon are activated by promoter hypomethylation in CML and that this event is frequently associated with the evolution of the disease to the advanced

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phase [5], suggesting that hypomethylation of repetitive sequences is a hallmark of CML progression.

In order to complete this attractive but incomplete picture, we have investigated the DNA methylation changes of the most important DNA repetitive elements during the progression of CML from chronic phase to blast crisis.

## 2. Materials and methods

### 2.1. Samples

Heparinized bone marrow cells were collected from patients with CML and from healthy marrow donors. $CD34^+$ cells were isolated using the MACS $CD34^+$ isolation kit (Miltenyi Biotec) and the AutoMACS selection device as described [6]. Paired samples, where both a CP diagnostic sample and a BC sample were available, were analyzed in 32 patients treated with interferon (22 in myeloid BC and 10 in lymphoid BC). BC was defined by the presence of at least 30% blasts in the blood or bone marrow or extramedullary involvement.

### 2.2. Semiquantitative real-time methylation-specific PCR (qrt-MSP) of repetitive sequences

We analyzed four repetitive sequences: L1, Alu, Satellite-alpha (Sat-a) and Satellite-2 (Sat-2). One microgram of genomic DNA was denatured by treatment with NaOH and modified by sodium bisulfite. qrt-MSP was performed as previously reported by our group [5] in a rapid fluorescent 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 $Mg^{2+}$ concentration in the reaction mixture was adjusted to 3.5 mmol/l. Amplification of the repetitive unmethylated sequences was used as target sequences. Primers for unmethylated reactions specific for the L1 sequences (L1-U) were based on a L1 consensus sequence (GenBank accession no. X52235). Primers for the Sat-a (Sat-a-U) and Sat-2 (Sat-2-U) unmethylated reactions were designed toward sequences specifically on chromosome 1 (GenBank accession nos. M38468 and X72623, respectively); however, satellite-specific sequences on other chromosomes may also be detected. We also used primers designed toward the unmethylated Alu consensus sequence (Alu-U) as previously reported [7]. Primer sequences are listed in Table 1.

The following program conditions were applied for qrt-MSP running: denaturation program, consisting in 1 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, 1 cycle at 95 °C for 0 s, 40 °C for 60 s and 90 °C for 0 s; and cooling program, 1 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 °C and 90 °C. Amplification of an Alu-control sequence (Alu-C) which is not modified by bisulfite treatment was performed as reference sequence [7]. It was amplified in the same run and following the same procedure described above for the different unmethylated repetitive sequences. A procedure based on the relative quantification of target sequence (Alu, L1, Sat-a and Sat-2 unmethylated sequences) versus their controls/calibrators in relation to the reference sequence (Alu-control sequences) was used to assess the degree of repetitive elements hypomethylation. Calculations were automatically performed by LightCycler software (RealQuant, version 1.0, Roche). The normalised ratio was obtained from the next equation and expressed as percentage of the control/calibrator:

$$\text{normalised ratio}\,(N_{\text{repetitive}}) = \frac{(E_{\text{target}})^{\Delta\text{Cp target (control}-\text{sample)}}}{(E_{\text{ref}})^{\Delta\text{Cp ref (control}-\text{sample)}}}$$

Efficiencies ($E$) of each gene were calculated from the slopes of crossover points (Cp) versus 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 hypomethylated patient but a relative value used as a measure for the relative level of repetitive elements hypomethylation in the particular sample). Water blanks were included with each assay. Results were confirmed by repeating bisulfite treatment and MSP assays for all samples.

### 2.3. Statistical analysis

All calculations were performed with the SPSS statistical package (SPSS, Chicago, IL). Mean normalised ratios and

Table 1
Sequences of the primers employed in this study

| | Forward primer (5′–3′) | Reverse primer (5′–3′) |
|---|---|---|
| LINE1-U | TTTATTAGGGAGTGTTAGATAGTGGGTG | CCTTACACTTCCCAAATAAAACAATACC |
| ALU-U | TGGTTAATATGGTGAAATTTTGTTTTTATT | TCCTACCTCAACCTCCCAAATAACT |
| SAT-A-U | TTGATGGAGTATTTTTAAAATATATGTTTTGTAGT | AAATTCTAAAAATATTCCTCTTCAATTACATAAA |
| SAT-2-U | TCGAATGGAATTAATATTTAACGGAAAA | CCATTCGAATCCATTCGATAATTCT |
| ALU-C | GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA | ATTAACTAAACTAATCTTAAACTCCTAACCTCA |