Brief communication

Methylation status of RASSF1A in patients with chronic myeloid leukemia

Antigoni Avramouli, Stefanos Tsochas, Eudokia Mandalas, Eirini Katodritou, Maria Ioannoud, Konstantinos Ritis, Matthaios Speletas

Abstract

RASSF1A, a key cell cycle related gene, is expressed in all hematopoietic cells, it is implicated in ras signaling pathway and its promoter hypermethylation is observed in a wide variety of solid tumors. Till now, RASSF1A methylation status has not been investigated in patients with chronic myeloid leukemia (CML). In this study, we analyzed 41 patients carrying the BCR-ABL rearrangement, in different stages of the disease. No patient displayed RASSF1A promoter methylation, although the K562 erythroleukemia cell line, bearing the BCR-ABL rearrangement, was found methylated. Thus, our findings indicate that RASSF1A methylation does not appear to represent a critical step in the pathogenesis and/or the progression of CML.

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1. Introduction

RASSF1A, a key cell cycle related gene, encodes a pro-apoptotic protein that contains a Ras-associated domain (RA domain). It is localized at 3p21.3 and its expression is silenced in a wide variety of solid tumors as a result, primarily, of promoter hypermethylation (reviewed by Donninger et al.) [1]. Interestingly, RASSF1A is expressed in all hematopoietic cells, modulates a broad range of cellular functions and its promoter methylation has been studied only in patients with acute leukemia and myelodysplastic syndromes, where it was found methylated only in a small proportion of them (ranging from 0% to 20%) [2,3]. However, the role of RASSF1A promoter methylation in chronic myeloid leukemia (CML) is still obscure.

CML is characterized by an expansion of myeloid, erythroid cells and platelets in peripheral blood and myeloid hyperplasia in the bone marrow. The hallmark of the disease is the Philadelphia (Ph) chromosomal (9;22)(q34;q11) translocation, producing a constitutively active tyrosine kinase protein, the p210bcr-abl [4].

Over the last few years, imatinib mesylate (Glivec, Novartis, Switzerland) has become the gold standard in the treatment of chronic myeloid leukemia (CML), and in the majority of cases, it is highly effective [4]. However, imatinib intolerance or resistance is increasingly recognized as a serious clinical problem, especially in patients with accelerated phase or blast crisis. Consequently, investigational treatments, such as the addition of demethylated agents have been tested [5], and found to be effective in a proportion of patients with resistant or progressive disease, suggesting that these could be used as an alternative treatment option [5]. Although several cell cycle related genes have been found hypermethylated in a proportion of CML patients during the progression of the disease (reviewed by Rush and Plass) [6], the molecular determinants of CML progression remain as yet elusive. Moreover, emerging evidence suggests that the N-ras pathway is constitutively active in CML, both in chronic phase and blast crisis, and considering that N-ras mutations are extremely rare [4–7], it is possible that other genes implicated in this pathway might be implicated in disease pathogenesis and progression, due to their genetic or epigenetic defect.

This study was scheduled in an attempt to investigate the RASSF1A methylation status in CML patients, both in chronic phase and during the progression of the disease, evaluating its possible prognostic and diagnostic significance.

2. Materials and methods

2.1. Patients

Forty-one patients (male/female: 21/20, mean age: 58.1 years, range: 28–80 years), suffered from CML at different stages of the disease, were enrolled in the study. In particular, 31 patients were analyzed at diagnosis, prior to imatinib treatment and among them 10 were reanalyzed, 8 during remission and 2 during progression of the disease to blast crisis. Archival bone marrow samples from 10 patients in chronic phase (1), accelerated phase (1), or blast crisis (8) were also...
examined. Twenty patients carried the b2a2-BCR-ABL rearrangement, 17 the b3a2-BCR-ABL, 2 both b3a2 and b2a2 and one the rare c3a2 (e19a2) rearrangement. One female patient during blast crisis displayed monosomy 7 (along with Ph chromosome), and another one—Y during remission of the disease (hematologic, complete cytogenetic and major molecular). At the end, the erythroleukemia cell line K562, carrying the b3a2-BCR-ABL rearrangement, with unknown RASSF1A methylation status, was also analyzed. DNA samples from patients with colon cancer and known methylation status were used as positive and negative controls, respectively. Moreover, 9 patients with non-Hodgkin lymphoma during remission of the disease were used as normal control group for mRNA expression assay.

The study was conducted in accordance with the principles of Helsinki declaration and was approved by the Institutional Review Board. All subjects gave informed consent and the procedures followed were in accordance with institutional guidelines.

2.2. DNA extraction, bisulfite treatment and methylation-specific PCR (MSP)

Genomic DNA was extracted from PB and/or bone marrow BM aspirates using the QIAamp DNA Blood Mini Kit (Qiagen) according to manufacturer's instructions. Archival bone marrow samples from 10 patients with CML were scraped from paraffin embedded sections and DNA was isolated using the QIAamp DNA FFPE Tissue Kit (Qiagen).

DNA methylation patterns in the CpG islands of the RASSF1A gene were determined as previously described [8], with some modifications. In particular, 1 µg genomic DNA was bisulfite modified by the EZ DNA methylation Gold KitTM (Zymo Research), according to the manufacturer's instructions. Modified DNA was then used in MSP for the detection of the methylation status of RASSF1A promoter. The primer sequences, designed to specifically methylated (M) or unmethylated (U) forms of the RASSF1A promoter were: M1 (forward) 5'-CGTTAAGCCGTCGCTACG-3'; M2 (reverse) 5'-AACCCCGGGAACTAAAAACAA-3'. For both PCR reactions, a total of 100 ng of modified DNA was amplified in a 25 µl reaction using 62.5 mM of each deoxyribonucleotide triphosphate, 20 pmole of each primer, 3.0 mM MgCl2 and 0.8 U Taq Polymerase (Qiagen) in a buffer supplied by the manufacturer. The conditions of both PCR were: 5 min at 94°C followed by 37 cycles (94°C for 30 s, 60°C for 30 s, 72°C for 30 s), and 4 min at 72°C after the last cycle. The M and U primer set generated a 94-bp and 108-bp products, respectively. PCR procedures were carried using the PCR-engine apparatus PTC-200, MJ-Research (Watertown-Massachusetts) and the PCR products were analyzed in 3% TBE agarose gels, stained with ethidium bromide, and visualized under UV light.

2.3. RNA extraction and quantitative real-time reverse-transcriptase PCR (Q-RT-PCR)

Total RNA was isolated from PB and BM aspirates from normal control group and 14 CML patients at diagnosis, 7 patients during remission of the disease and 4 patients at blast crisis, using TRIzol (Ambion, Austin, USA). The mRNA levels of RASSF1A were determined in a Q-RT-PCR using SYBR-Green PCR Supermix (Invitrogen, UK), in the automated thermocycler RotorGene 6000 (Corbett Life Science, Sydney, Australia). An 1/20 aliquot of the cDNA reaction product was used in duplicate Q-RT-PCR reactions and all measurements were averaged. Primers used (forward 5'-TCACTGCGGGTTGTCGCT-3'; reverse 5'-CTTCGCTGTCGCTACG-3') are available online at "http://medgen.ugent.be/rtprimerdb/". Thermocycler conditions included an initial holding at 95°C for 2 min and subsequently at 95°C for 2 min, followed by 45 cycles at 95°C for 10 s, 62°C for 10 s and 72°C for 30 s. The beta-2-microglobulin (B2M) gene was used as an endogenous control for sample normalization (reference gene), and its primers were commercially obtained by SA Biosciences (USA). Thermocycler conditions for B2M included an initial holding at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s and 60°C for 30 s. The efficiency of Q-RT-PCR reactions was 0.95 and 1.02 for RASSF1A and B2M genes, respectively. Relative quantification and calculation of the range of confidence were performed using the comparative DDCT method, as described previously [9]. The relative expression of RASSF1A was presented as a multiple of the respective gene expression in a normal control sample selected randomly. Statistical analysis was performed using the REST (relative expression software tool) statistical software. A p-value of <0.05 was considered statistically significant.

3. Results

Methylation of RASSF1A was detected in none of the CML patients at chronic phase (33 samples), at remission (8 samples) or during the progression of the disease (1 sample in accelerate phase and 10 samples in blast crisis). Interestingly enough, the K562 cell line was found methylated. Examples of methylation status of patients' and cell line samples are presented in Fig. 1.

The results of RASSF1A methylation status were indirectly confirmed by Q-RT-PCR analysis. In particular, we did not observe any significant difference on RASSF1A mRNA expression between patients at diagnosis vs normal controls (mean ± S.D: 0.84 ± 0.6 vs 0.85 ± 0.6, p = 0.996), between patients at diagnosis vs patients at blast crisis (0.84 ± 0.6 vs 1.2 ± 0.8, p = 0.829) and between patients at diagnosis vs patients during remission of their disease (0.84 ± 0.6 vs 0.67 ± 0.4, p = 0.988). Interestingly, patients with colon cancer with methylated RASSF1A display a 3–6-fold reduction of gene expression compared to adjacent normal tissues (manuscript in preparation).

4. Discussion

In this study, we analyzed patients with CML in different stages of the disease but no methylation of RASSF1A promoter was observed. Moreover, we also analyzed a patient with the rare c3a2 (e19a2) BCR-ABL rearrangement, encoding the p230bc-abl protein, but no RASSF1A promoter methylation was found. To the best of our knowledge this is the only report describing the methylation status in CML. Interestingly, Jost et al. have recently observed that RASSF1A methylation was also absent in BCR-ABL negative myeloproliferative diseases (MPDs) [10] and in conjunction with our findings indicate that RASSF1A silencing may not represent a critical step in the pathogenesis of MPDs.

Nevertheless, we found RASSF1A promoter methylation in K562 cell line, which is an erythroleukemia cell line bearing the BCR-ABL rearrangement. We cannot exclude the possibility that this finding might be the result of intense culture conditions. However, previous studies have shown that some patients with acute leukemia display RASSF1A methylation [1], and possibly the deactivation of RASSF1A in K562 may account in this context.

In conclusion, although RASSF1A is methylated in a wide range of solid tumors and it is expressed in all hematopoietic cells, our results indicate that its promoter methylation does not participate in the pathogenesis and the progression of CML.

Conflict of interest

None.

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References