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Expression of the cell cycle regulators $p14^{ARF}$ and $p16^{INK4a}$ in chronic myeloid leukemia

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Abstract

Expression of $p14^{ARF}$ and $p16^{INK4a}$ tumor suppressor genes was investigated in 109 patients with chronic myeloid leukemia (CML). The $p14^{ARF}$ and $p16^{INK4a}$ mRNA levels were significantly low in patients in chronic phase (CP) at presentation and high in patients treated with interferon-α (IFN-α), especially in non-responders. A moderate overexpression of $p14^{ARF}$ with a normal expression of $p16^{INK4a}$ was observed in imatinib-resistant patients. Although protein expression did not consistently match mRNA levels, a role for the two cell cycle regulators in the IFN-α signaling pathway is suggested as well as a relation with the resistance to IFN-α or imatinib therapy.

Keywords: $p14^{ARF}$; $p16^{INK4a}$; CML; Q-RT-PCR; Gene expression

1. Introduction

The INK4a locus located on chromosome 9p21 plays a major role in the pathogenesis of several types of malignant disorders [1,2]. It encodes cell cycle inhibitors $p14^{ARF}$ ($p19^{ARF}$ in mice) and $p16^{INK4a}$ that control the p53 and retinoblastoma (pRb) pathways, respectively [3]. The $p14^{ARF}$ protein suppresses cell growth through multiple p53-dependent or -independent pathways. In particular, $p14^{ARF}$ stabilizes p53 by inhibiting Mdm2-dependent p53 degradation [4]. The $p16^{INK4a}$ protein prevents phosphorylation and inactivation of the pRb protein through inhibition of the cyclin D-dependent kinases CDK4 and CDK6 [5]. Thus, INK4a locus regulates two pathways that are crucial in the maintenance of cellular homeostasis and in the prevention of oncogenic processes.

The $p14^{ARF}$ and $p16^{INK4a}$ tumor suppressor genes are often inactivated in human cancers as a result of gene deletions, point mutations or promoter methylation [6–8]. Deletions of the INK4a locus are well documented in hematological malignancies, mainly in T-cell acute lymphoblastic leukemia [9]. On the other hand, very few studies are available with regard to the expression of these genes at the transcriptional level in hematological malignancies. In this study, we investigated the expression of $p14^{ARF}$ and $p16^{INK4a}$ genes in chronic myeloid leukemia (CML), a malignant hematopoietic stem cell disorder characterized by the Ph1 chromosome and its molecular counterpart, the BCR-ABL fusion gene, which is translated into a functional protein [10,11]. The Bcr-Abl oncoprotein is directly responsible for the initial manifestations of the disease through its constitutive tyrosine kinase activity. CML progresses from a chronic phase

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(CP) to an accelerated phase (AP) and a final blast crisis (BC). Major treatment options are allogeneic hematopoietic stem cell transplantation, cytokines such as interferon-alpha (IFN-α), or imatinib mesylate, the latter being currently the new "gold standard". The main aim of the study was to evaluate $p14^{ARF}$ and $p16^{INK4a}$ mRNA expression levels in treated and untreated CML patients during different clinical phases of the disease using quantitative-reverse transcription-PCR (Q-RT-PCR).

2. Patients and methods

2.1. Patients

We retrospectively analyzed 210 blood samples from 109 CML patients followed in our institution between 2000 and 2005. At the time of the study, median age was 60 years (range 16–80); 56% were men and 44% were women (Table 1). According to their clinical and biological status and treatment at the time of analysis, several groups of patients have been identified, including patients in CP at presentation before any treatment, patients in AP or BC, patients in long complete cytogenetic remission (CCR) after cessation of IFN-α therapy, patients treated with IFN-α and patients treated with imatinib mesylate (Table 1). The two latter groups were subdivided in two categories according to the response to treatment. Imatinib was used either as initial treatment or after failure of IFN-α therapy. Ten IFN-α non-responder patients out of 13 received imatinib and were submitted to a long-term molecular follow-up. Cytogenetic responses were assessed in every patient at 3, 6 and 12 months of therapy during the first year and every 6 months thereafter. BCR-ABL transcript levels in peripheral blood samples were quantified by Q-RT-PCR every 3 months. Median levels of Ph+ cells and BCR-ABL transcripts are indicated in Table 1. The lowest BCR-ABL mRNA levels were found in patients in prolonged CCR either after cessation of IFN-α or under IFN-α or imatinib therapy (responders). The persistence of blood cells expressing the BCR-ABL chimeric mRNA, even at extremely low levels, in spite of normal cell counts and of the absence of detectable Ph+ chromosome, underlines the potential risk of relapse.

As controls for the normal expression of $p14^{ARF}$ and $p16^{INK4a}$, blood samples from 32 healthy blood donors were used (normal controls). Negative controls included two samples from the K562 cell line, which defects the INK4a locus [12]. All patients and blood donors provided informed consent for participation in the study according to the declaration of Helsinki.

2.2. RNA preparation and cDNA synthesis

Total RNA was extracted from blood samples or from the K562 cell line with RNABle reagent (Eurobio, Les Ulis, France). RNA pellets were resuspended in 40 μl RNAse-free water and quantified by UV spectrophotometry. A 1 μg of total RNA was reverse-transcribed in 20 μl with 1 mM of each dNTP, 25 μM of random hexamers, 20 units of RNAse Out and 100 units of M-MLV Reverse Transcriptase (Invitrogen, Cergy-Pontoise, France) in 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT.

2.3. Quantitative RT-PCR

mRNA levels were determined by Q-RT-PCR using the 7000 Sequence Detection System and the human p14 and p16 TaqMan pre-developed assay reagents (Applied Biosystems, Foster City, CA, USA). $p14^{ARF}$ and $p16^{INK4a}$ expressions were normalized by measuring ABL mRNA levels in the same cDNA samples (internal reference). ABL mRNA amplification was performed as recommended by the European Against Cancer collaborative group [13]. Q-RT-PCR reactions were prepared in duplicates in a final volume of 25 μl using the TaqMan Universal PCR Master Mix 2X (Applied

Table 1
Characteristics of CML patients and controls

Subjects under study	Treatment at the time of analysis	No. of patients [men; women]	No. of samples	Median age at analysis	Median % of Ph+ cells	Median % BCR-ABL/ABL
Normal controls (healthy blood donors)			32			
CML patients at presentation (CP)	No treatment	11 [7; 4]	11	60	100	82.5
CML patients in AP or BC	Cytotoxic chemotherapy	17 [11; 6]	17	55	100	65
CML patients in long CCR after cessation of IFN-α	No treatment	9 [6; 3]	22	60	0	0.11
CML patients treated with IFN-α[a]						
IFN-α responders	IFN-α	10 [4; 6]	23	49	0	0.18
IFN-α non-responders	IFN-α	13 [7; 6]	13	61	100	77
CML patients treated with imatinib[b]						
Imatinib-sensitive	Imatinib	22 [16; 6]	61	65	0	0.077
Imatinib-resistant	Imatinib	27 [10; 17]	63	61	100	55.8

[a] Patients treated with IFN-α2a or 2b at the initial dose of 5 MU/m^2 daily subcutaneously, the dose being adjusted according to side effects, hematological parameters and cytogenetic response.
[b] Patients treated with imatinib mesylate at the initial dose of 400 mg daily.