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Expression of cell cycle regulatory genes in chronic myelogenous leukemia

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ABSTRACT

Background and Objective. **Cell cycle regulatory genes are frequently altered in a variety of malignancies. The structure and pattern of expression of eight genes involved in cell division cycle control were studied in leukemic cell samples prepared from bone marrow of patients affected by chronic myelogenous leukemia.**

Design and Methods. **Ten cell preparations were obtained from patients in the chronic phase, five from those in myeloid blast crisis and five from those in the lymphoid acute phase. Moreover, bone marrow $CD34^+$ cells, purified from healthy subjects and patients with chronic myelogenous leukemia (both during chronic and acute phases), were analyzed. The investigated genes were RB1, p53 and six cyclin-dependent kinase inhibitor genes ($p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, $p21^{WAF1/CIP1}$, $p27^{Kip1}$, $p57^{Kip2}$).**

Results. **We found that none of these genes is structurally altered in either the chronic or acute phases, with the single exception of the $p16^{INK4A}$ gene, which was homozygously deleted in 1 case of lymphoid evolution. $p57^{Kip2}$ expression is down-regulated during the evolution towards the blast crisis both in malignant and $CD34^+$ cells. In addition, a significant up-regulation of $p15^{INK4B}$ gene expression is observable during the development of the acute phase of malignancy.**

Interpretations and Conclusions. **The transcriptional modulation of some cyclin-dependent kinase inhibitors might contribute to the fatal blast crisis of chronic myelogenous leukemia.**

Key words: cell cycle, leukemia, chronic myelogenous leukemia, tumor suppressor genes, CDKNI

Chronic myelogenous leukemia (CML) is a hematologic stem cell disorder characterized by two distinct clinical phases: an initial chronic phase lasting from 2 to 4 years, followed by a fatal blast crisis.[1] The major abnormality of the chronic phase is marked expansion of the stem cell compartment committed to myelopoiesis. The acute phase (blast crisis) is characterized by a decrease in the differentiation ability of the cells and by the loss of response to regulatory factors.[2]

The cytogenetic hallmark of the leukemic clone is the Philadelphia chromosome, a balanced reciprocal translocation which involves the *c-abl* proto-oncogene on chromosome 9 and the *bcr* gene on chromosome 22.[3,4] The chimeric *bcr-abl* gene generally transcribes a 8.5 kb hybrid mRNA which results in a 210 kD fusion protein (P210).[5,6] Recently, it has been shown that P210 inhibits the apoptotic program, which is activated by DNA damage.[7] Eukaryotic cells respond to genomic alteration(s) by blocking cell cycle progression at both G1/S and G2/M transitions,[8] thus allowing DNA repair. The late-G1 phase arrest is due to p53-mediated induction of $p21^{WAF1/CIP1}$, a powerful cyclin-dependent kinase (CDK) inhibitor.[9] On the other hand, if DNA synthesis proceeds in the presence of damaged DNA, p53 triggers cell death by apoptosis. Since this mechanism should avoid the accumulation of genetic alterations, its impairment by P210 might be important in CML progression.

Although it has been definitely demonstrated that the *bcr-abl* fusion gene plays a central role in the pathogenesis of the chronic phase, the additional genetic and molecular events leading to CML blast crisis are still poorly understood. Moreover, since CML evolution occurs toward two distinct forms, namely a myeloid or a lymphoid acute phase, different genetic alterations might lead to the distinct blast crisis phenotypes.

So far few gene alterations have been shown to occur in the CML acute phase. In particular, p53 deletions and/or mutations have been described in only 10-25% of the cases of CML;[10] molecular abnormalities involving the RB1 tumor suppressor gene and the RAS family of proto-oncogenes have occasionally been reported.[11-13]

Recently, biochemical and genetic studies suggest that a new family of cell cycle regulatory proteins, called CDK inhibitors (CDKI), may act as tumor suppressor factors.[14] These proteins bind to and inhibit specific CDKs or CDK-cyclin complexes, thereby

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resulting in arrest of the cell cycle at the late-G1 checkpoint. Thus, their inactivation consistently increases the rate of cell proliferation and the development and/or progression of transformed clones.

Two classes of CDKI genes have been identified: the INK4 gene family (including $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, $p19^{INK4D}$), and the CIP/Kip gene family ($p21^{WAF1/CIP1}$, $p27^{Kip1}$, $p57^{Kip2}$). Among these CDKIs, both $p16^{INK4A}$ and $p15^{INK4B}$ genes are frequently homozygously co-deleted in transformed cell lines as well as in many human cancers.[15,16]

Although previous studies have demonstrated the loss of function of CDKI tumor-suppressor genes in human cancers, the structural and functional status of these genes have not been investigated in detail in CML during chronic and acute phases. Interestingly, we[17] and others[18] demonstrated that homozygous deletions of $p16^{INK4A}$ gene occur in from 18% to 50% of cases with lymphoid blast crisis of CML, thus suggesting the presence of distinct pathways in the pathogenesis of lymphoid and myeloid blast crises of CML.

To unravel the role of cell cycle-control genes in CML, we analyzed the expression of most of them ($p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, $p21^{WAF1/CIP1}$, $p27^{Kip1}$, $p57^{Kip2}$, p53 and RB1) in 20 malignanant cell samples (10 chronic phases, 5 myeloid blast crises, 5 lymphoid blast crises) obtained from bone marrow. This investigation was carried out to evaluate the incidence of inactivation of these TSGs in CML, and the possible correlation between their expression and the progression from a relatively benign chronic phase to an aggressive and terminal acute phase. Moreover, in order to shed some light on the complex situation occurring at bone marrow level during the evolution of CML, we prepared and characterized the $CD34^+$ cell fraction (which contains stem cells) from normal subjects and patients affected by CML at different clinical stages.

Materials and Methods

Patients and samples

Twenty bone marrow samples were obtained from 19 patients with CML (10 chronic phases, 5 myeloid blast crises, 5 lymphoid blast crises); samples #3 and #17 were collected from the same patient. Blastic phase was established according to Arlin *et al.*,[19] and the immunophenotypic characterization of the cells was performed as previously described elsewhere.[20] Standard methods for cytogenetic and molecular analysis (*bcr/abl* rearrangement) have been previously reported.[21] In order to minimize the possibility of contamination of the blast population with more mature cells, only samples showing a percentage of blasts >90% were included in this study; finally the blast population was further enriched with a Ficoll-Hypaque density gradient separation (final preparations, between 98-100% purity). $CD34^+$ enriched cell preparations were obtained as previously described.[22]

High molecular weight DNA was purified by protein digestion with proteinase K, extraction by phenol-chloroform and precipitation by ethanol as previously described.[23]

Cyclin-dependent kinase inhibitor gene (CDKI) genomic assay was performed by amplifying exon 1 and 2 of the relative genes as described elsewhere.[24] Total RNA was isolated from samples by guanidium-thiocyanate.[25]

Reverse transcriptase (RT)-PCR

Total RNA (1 µg) was reverse transcribed by using Superscript II RNase H (Gibco-BRL) reverse transcriptase (200 units). cDNA samples were amplified by PCR using 200 ng of template, 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 20 pmol of each primer, 200 µmol of each nucleotide and 1 U Taq DNA polymerase.

We studied the expression of the following genes: $p15^{INK4B}$, $p16^{INK4A}$ (a and b transcripts), $p18^{INK4C}$, $p21^{WAF1/CIP1}$, $p27^{Kip1}$, $p57^{Kip2}$, p53 and RB1 by using the primers showed in Table 1. Moreover, the transcription of cyclin D1, D2 and D3 was investigated in $CD34^+$ cell preparations as described elsewhere.[26]

PCR conditions were: 30-35 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, for the majority of examined genes. The annealing temperatures were 58°C for $p18^{INK4C}$ and $p57^{Kip2}$, 53°C for RB1 and 50°C for p53 gene.

Before amplification with the specific primers, an aliquot of the cDNA preparation was amplified using glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers (Table 1) to determine the efficacy of generated cDNA. Moreover, we used three different cDNA concentrations to ensure that signals were proportional to input mRNA, and each experiment was performed at least in duplicate and several in triplicate. Finally, G3PDH cDNA was co-amplified with each CDKI as an internal control of the reaction.

Aliquots of PCR reaction (5 µL) were separated by non-denaturing 8% polyacrylamide gel and stained by the standard silver nitrate method.

Results

Analysis of $CD34^+$ cell preparations

As reported in Table 2, the expression of 13 genes related to the control of the cell division cycle was studied in $CD34^+$ cell preparations generated from bone marrow of three normal subjects, two patients with chronic phase CML, and two patients with blast crisis CML. The genes analyzed were RB1, p53, seven CDKI and all members of the cyclin D family (see Table 2). Normal $CD34^+$ cells express all the CDKI analyzed except $p16^{INK4A}$ (both a and b transcripts). Moreover, in these cells, cyclin D2 and D3 genes are transcribed while cyclin D1 gene is not expressed. This pattern perfectly matches the results of a detailed

Table 1. List of primers used in RT-PCR reaction.

Amplimers for p15^{INK4B}:	
Direct	5'GGTAAGAAAATAAAGTCGTTG 3'
Forward	5'GGAATGCGCGAGGAGAACAAGGGCATG '3
Amplimers for p16^{INK4A}(a-transcript):	
Direct	5'GGGTAGAGGAGGTGCGGGC 3'
Forward	5'GCGATGGCCCAGCTCCTC 3'
Amplimers for p16^{INK4A}(b-transcript)	
Direct	5'TACTGAGGAGCCAGCGTCTA 3'
Forward	5' AGCACCACCAGCGTGTC 3'
Amplimers for p18^{INK4C}	
Direct	5'TGATCGTCAGGACCCTAAAG 3'
Forward	5'CTGCAGCGCAGTCCTTCC 3'
Amplimers for p21$^{WAF1/CIP1}$	
Direct	5'AGGCGCCATGTCAGAACCGGCTGG 3'
Forward	5'GGAAGGTAGAGCTTGGGCAGGC 3'
Amplimers for p27^{Kip1}	
Direct	5'ATGTCAAACGTGCGAGTGTCTAAC 3'
Forward	5'TTACGTTTGACGTCTTCTGAGGCCA 3'
Amplimers for p57^{Kip2}	
Direct	5'TCCACGATGGAGCGTCTTGT 3'
Forward	5'GTCCACTTCGGTCCACTGCA 3'
Amplimers for *p53*	
Direct	5'AAGTCTGTGACTTGCACG 3'
Forward	5'CTGGAGTCTTCCAGTGTG 3'
Amplimers for *RB1*	
Direct	5'GATAATGCTATGTCAAGACT 3'
Forward	5'CAAGGACACATAGCATTAAC 3'
Amplimers for G3PDH	
Direct	5'GGTATCGTGGAAGGACTCATGAC 3'
Forward	5'ATGCCAGTGAGCTTCCCGTCAGC 3'

immunochemical investigation,[22] thus demonstrating in this cell population a strict correspondence between mRNA and protein analyses, confirming the value of mRNA studies.

The analysis of CD34$^+$ fractions from chronic and acute CML, compared to normal samples, revealed: i) the down-regulation of p57^{Kip2} transcription during the acute evolution; p57^{Kip2} was indeed detectable only in CD34$^+$ cells from control and chronic CML, and ii) the p15^{INK4B} expression in normal and acute phase CML CD34$^+$ cells, but not in chronic phase CML (Table 2). Conversely, a complex and not well-defined expression pattern was observed in the case of p16^{INK4A}, while the expression of the other genes is similar to that in CD34$^+$ cells from normal and CML subjects.

Analysis of malignant cells from chronic myelogenous leukemia at different clinical stages

p16^{INK4A}, p15^{INK4B} and p18^{INK4C} gene expression

p16^{INK4A}, p15^{INK4B} and p18^{INK4C} mRNA occurrence was investigated in 20 patients with CML (10 chronic phases, 5 myeloid blast crises, 5 lymphoid blast crises) (Table 3). p16^{INK4A} mRNA was detected in 4 out of the 10 chronic phases, whereas none of the cases with lymphoid blast crisis and only one of the patients with myeloid blast crisis expressed it.

p15^{INK4B} mRNA was observed more frequently during blast crises (80%) than during chronic phases (30%) (Table 2 and Figure 1). Interestingly, when leukemic cells were obtained during the chronic phase (#3) and during the blast crisis (#17) from the same patient, a different picture was observed for p16^{INK4A} and p15^{INK4B} mRNA. Indeed, while p16^{INK4A} expression was undetectable in both disease stages, p15^{INK4B} expression was found only in the acute phase.

It is to underline that DNA analysis by PCR of p16^{INK4A} and p15^{INK4B} exons 1 and 2 demonstrated the occurrence of normal genes in all cases with the exception of sample #20, (lymphoid blast crisis) where p16^{INK4A} gene deletion was observable in the presence of a normal p15^{INK4B} gene.

We also investigated the expression of the alternative p16^{INK4A} transcript (the so-called b-transcript) (Table 2). In 16 out of the 20 CML samples (80%) we

Table 2. Expression of cell division cycle genes in CD34$^+$ cells from normal subjects and patients affected by chronic myelogenous leukemias.

	G3PDH	*p16a*	*p16b*	*p15*	*p18*	*p21*	*p27*	*p57*	*RB1*	*p53*	*CycD1*	*CycD2*	*CycD3*
Control subjects													
1	+	-	-	+	+	+	+	+	+	+	-	+	+
2	+	-	-	+	+	+	+	+	+	+	-	+	+
3	+	-	-	+	+	+	+	+	+	+	-	+	+
CML- chronic phase													
1	+	-	±	-	+	+	+	+	+	+	-	+	+
2	+	-	-	-	+	+	+	+	+	+	-	+	+
CML, blast crisis													
1*	+	-	-	+	+	+	+	-	+	+	-	+	+
2°	+	±	-	+	+	+	±	-	+	+	-	+	+

*CycD1, CycD2 and CycD3: Cyclin D1, D2 and D3, respectively. *myeloid blast crisis; °lymphoid blast crisis.*

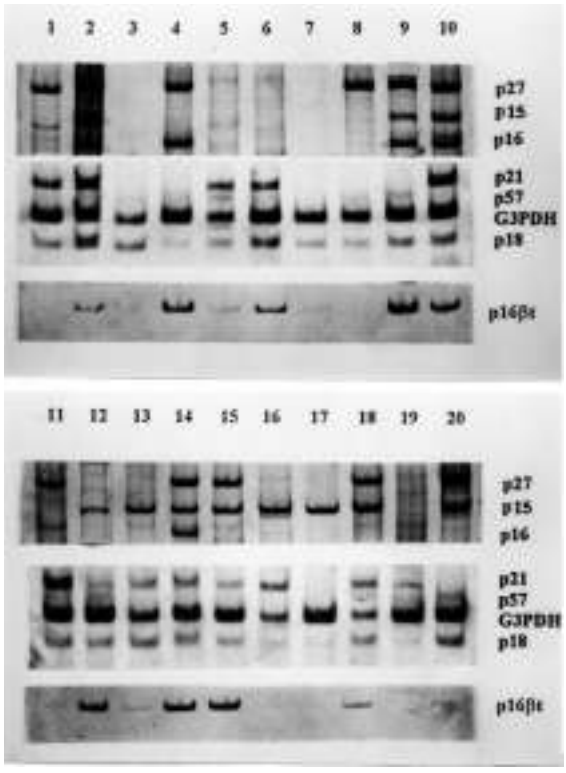

Figure 1. Reverse-transcriptase PCR of the CDKI (p15^{INK4B}, p16^{INK4A} a and b transcripts, p18^{INK4C}, p21$^{WAF1/CIP1}$, p27^{Kip1}, p57^{Kip2}) and of G3PDH fragments. Lanes 1-10 = patients in chronic phase; lanes 11, 12, 13, 14 and 19 = patients in myeloid blastic crisis; lanes 15, 16, 17, 18 and 20 = patients in lymphoid blast crisis.

observed a perfect agreement between a- and b-form transcription. However, in 4 out of 20 cases we found expression solely of the b-transcript. These findings suggest the occurrence of distinct regulatory mechanisms and/or of a transcriptional block by the 5'CpG island methylation of the a-transcript promoter region. Finally, p18^{INK4C} mRNA expression was found in all examined cases (Table 3).

p21$^{WAF1/CIP1}$, p27^{Kip1} and p57^{Kip2} genes expression

As shown in Table 3, RT-PCR analysis of the 20 tested specimens detected p21$^{WAF1/CIP1}$ expression more frequently in acute than in chronic phases (5/10 chronic phases, 5/5 myeloid blast crises, 3/5 lymphoid blast crises).

Moreover, p27^{Kip1} mRNA expression was found in the majority of the chronic cases analyzed (6/10) and 50% of blast crises (2/5 myeloid blast crises, 3/5 lymphoid blast crises). Finally, we also investigated p57^{Kip2} gene expression. Our findings indicate a clear decrease of its expression during acute evolution (80% versus 50%).

p53 and RB1 gene transcription

Clearly detectable levels of p53 mRNA were found in all the analyzed cases except two patients with lymphoid blast crisis (Table 3). Expression of RB1 mRNA was also evaluated because the absence of pRb protein was reported to occur frequently with structurally intact RB1 gene in Southern blot analysis.[27] RB1 mRNA was expressed in all the specimens.

Discussion

Malignant transformation might be viewed as a multistep process in which accumulated genetic changes lead to an unregulated cell clonal expansion which progressively evolves toward a more aggressive phenotype.[28] Among the numerous molecular mechanisms altered in human neoplasias, those involving cell division cycle control are believed fundamental for carcinogenesis.[16] Indeed, many (and probably all) human cancers present abnormalities in one or more of genes regulating cell proliferation, including *RB1, p53* and the recently isolated CDKI genes. These concepts are well illustrated by CML, which: i) derives from cycling stem cells capable of re-entering G0 phase and of resuming cell cycle steady-state kinetics, and ii) evolves from a chronic phase toward an acute fatal blast crisis.[1,2]

In CML, it has been demonstrated that molecular events other than the transcription of the BCR/ABL (*bcr-abl*) chimeric gene may be responsible for the evolution of the chronic phase. However, studies on *p53, RB1* and *RAS* suggest that these genes are rarely altered in chronic or acute CML.[11,29-31] Moreover, homozygous deletion of p16^{INK4A} gene, which is a very frequent event in multiple human tumors, seems to occur solely in some cases of lymphoid CML evolution.[17,18]

Although previous studies have analyzed the structural status of *p53*, *RB1* and a few CDKI in CML, the expression level of these TSGs has not been investigated. However, this information could have remarkable value for understanding the events leading to the fatal blast crisis. Therefore, we decided to examine the expression of several tumor suppressor genes (p15^{INK4B}, p16^{INK4A}, p18^{INK4C}, p21$^{WAF1/CIP1}$, p27^{Kip1}, p57^{Kip2}, *p53* and *RB1*) in chronic and acute CML. To this aim, we prepared highly purified malignant cell populations (>95%) from bone marrow of CML patients and analyzed the expression of the above reported genes by a semi-quantitative RT-PCR methodology.

A major problem in drawing conclusions from these studies is the difficulty in comparing malignant cells from the chronic phase with those from the blastic phase. Thus, we decided to analyze CD34$^+$ preparations from bone marrow of normal subjects as well as of patients affected by CML at different stages.

Our data show that p16^{INK4A} a-transcript is undetectable in 60% chronic phase samples and in 90% of acute phase specimens. Interestingly, none of our 5

Table 3. Expression of CDki (p16^{ink4a}, p15^{ink4b}, p18^{ink4c}, p21$^{waf1/cip1}$, p27^{kip1}, p57^{kip2}), rb1 and p53 in chronic and acute phases of chronic myelogenous leukemia.

	G3PDH	p16a	p16b	p15	p18	p21	p27	p57	RB1	p53
Chronic phase										
1	+	-	-	-	+	+	+	-	+	+
2	+	+	+	+	+	+	+	+	+	+
3	+	-	-	-	+	-	-	+	+	+
4	+	+	+	-	+	-	+	+	+	+
5	+	-	-	-	++	+	-	+	+	+
6	+	-	+	-	+	+	-	+	+	+
7	+	-	-	-	+	-	-	-	+	+
8	+	-	-	-	+	-	+	+	+	+
9	+	+	+	+	+	-	+	+	+	+
10	+	++	++	+	+	+	+	+	+	+
Blast crisis, myeloid										
11	+	-	-	-	+	+	+	+	+	+
12	+	-	+	+	+	+	-	+	+	+
13	+	-	-	+	+	+	-	-	+	+
14	+	+	+	+	+	+	+	+	+	+
15	+	-	-	-	++	+	-	-	+	+
Blast crisis, lymphoid										
16	+	-	+	+	+	+	+	-	+	+
17	+	-	-	+	+	+	-	-	-	+
18	+	-	-	+	+	-	-	-	-	+
19	+	-	-	+	+	-	+	+	+	+
20	+	-	+	+	+	+	+	+	+	+

lymphoid blast crisis specimens expressed this gene. In addition, this gene was not expressed in any CD34$^+$ cell preparations investigated. The function of p16^{INK4A} gene has been recently demonstrated to be linked to the physiological ageing process of cells.[32] Thus, the absence of transcription of this gene in CD34$^+$ preparations might be due to the role of this cellular population as a stem compartment. Conversely, the occurrence of p16^{INK4A} transcripts in the chronic phase (opposed to its absence in the blastic phase) might be related to the low cycling rate (and, possibly, ageing) of these cells.

The absence of p16^{INK4A} transcripts might be due to different mechanims including: i) homozygous deletions; ii) point mutations and iii) transcriptional silencing. One case out of the 5 lymphoid blast crises had p16^{INK4} deletion. However, no point mutations have been demonstrated in human acute or chronic hematologic tumors.[23,33-35]

More recently, an additional mechanism of p16^{INK4A} gene inactivation, namely transcriptional block by methylation of the 5'-CpG islands, has been described in various human tumors.[36,37] This phenomenon appears to be highly selective since it depends on cancer type. Indeed, in most epithelial-derived tumors the DNA methylation-associated inactivation occurs exclusively at the p16^{INK4A} gene while in gliomas and leukemias (expecially acute myeloid leukemias) this mechanism seems to involve the p15^{INK4B} gene.[37]

Our findings suggest that the inactivation of p16^{INK4A} gene transcription established in 60% chronic CML and 90% acute CML might be important in this tumor and possibly in the evolution of the disease. The absence of p16^{INK4A} a-transcript could have been mostly due to selective hypermethylation since in some cases the alternative p16^{INK4A} b-transcript was observable. Moreover, in 1 out of 5 lymphoid blast crises analyzed, p16^{INK4A} was inactivated by homozygous deletions thus confirming the role of this genetic alteration in such CML progression.

p15^{INK4B} was expressed in the majority of CML analyzed (11/20), more frequently in the blast (8/10) than in the chronic phase (3/10) (Table 3). This result was confirmed by repeated experiments, employing different PCR conditions and amounts of cDNA. Moreover, this finding was strongly confirmed by the analysis of the CD34$^+$ cell samples (Table 2). Indeed, CD34$^+$ cells from normal subject transcribed this gene, as also demonstrated by a recent analysis at protein level.[22] Conversely, CD34$^+$ cells from chronic CML (analogously to malignant cells of the same stage of the tumor) did not express this gene. Finally, the CD34$^+$ cells from CML acute phase (like blast preparations of the same tumor stage) actively transcribed the gene.

The increased expression of p15^{INK4B} observed during the transition toward the blast crisis phase might appear difficult to explain. However, it is interesting to note a similar condition has been observed in human neuroblastoma. Indeed, it was recently demonstrated that p15^{INK4B} expression increased in neuroblastoma with a worst prognosis (stage IV) compared to neuroblastoma with a better evolution (stages I, II).[38] Thus, the presence of specific p15^{INK4B} RNA in cells of blast crisis might represent a phenotypic feature of cycling cells or an attempt to slow

the rate of growth.

An important result of this study is the increased silencing of $p57^{Kip2}$ gene from the chronic form (20%) towards the acute phase (50%) (Table 3). Importantly, a superimposable finding was obtained analyzing $CD34^+$ populations (normal + chronic phase versus blastic phase). The present investigation, to the best of our knowledge, is the first analysis of the expression of this gene in human blood malignancies and one of the first on human cancers. $p57^{Kip2}$ gene maps at a very interesting genetic locus, which contains several (including $p57^{Kip2}$ gene itself) imprinted genes.[39,40] Thus, the observed transcriptional regulation is particularly intriguing due to the increasing importance given to the imprinting phenomenon in the development of human cancers.

In summary, our data allow us to conclude that, at variance to other conditions,[41] none of the genes whose expression was investigated seems to be structurally altered in human chronic phase or acute phase CML. The only exception is the $p16^{INK4A}$ gene, which is homozygously deleted during lymphoid CML evolution.[17,18] However, at least for three genes, i.e. $p15^{INK4B}$, $p57^{Kip2}$, and, possibly $p16^{INK4A}$, the transcriptional regulation seems correlated to the evolution of human CML. Further studies, mainly devoted to manipulation of the CDKIs content, are required to unravel the role of these genes in the development of human CML blastic crisis.

Contributions and Acknowledgments

AI was the principal investigator, designed the study and was responsible for data handling and interpretation and writing of the paper. FDR carried out Western blot analysis and collaborated in the writing. BG, AS and MFF prepared samples and blast cells, they all performed genomic and expression analysis studies. GS was responsible for ethical approval, recruitment of patients, day-to-day contact with partecipants. All the authors gave their critical contribution to the manuscript and approved the final version.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

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