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Frequent methylation of $p16^{INK4A}$ and $p14^{ARF}$ genes implicated in the evolution of chronic myeloid leukaemia from its chronic to accelerated phase

E. Nagy[a], Z. Beck[a], A. Kiss[b], E. Csoma[a], B. Telek[b], J. Kónya[a], É. Oláh[c], K. Rák[b], F.D. Tóth[a,*]

[a]*Institute of Medical Microbiology, Medical and Health Science Center, University of Debrecen, Debrecen, Nagyerdei krt. 98, H-4012 Debrecen, Hungary*

[b]*2nd Department of Medicine, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary*

[c]*Department of Pediatrics, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary*

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Abstract

The frequency and mechanism of $p16^{INK4A}$ and $p14^{ARF}$ gene alterations were studied in cell samples from 30 patients with Philadelphia (Ph) chromosome-positive chronic myeloid leukaemia (CML), both at diagnosis and at the onset of the accelerated phase (AP) of the disease. No alterations in the $p16^{INK4A}$ or $p14^{ARF}$ genes were found in any of the chronic phase (CP) samples. DNA sequencing analyses detected $p16^{INK4A}$ or $p14^{ARF}$ mutations in 17 AP samples. All mutations were heterozygous without loss of the other allele. Aberrant methylation of the $p16^{INK4A}$ or $p14^{ARF}$ promoters was found in 14 of 30 AP samples. The most common situation was the simultaneous methylation of both promoters. Our data indicate that $p16^{INK4A}$ and $p14^{ARF}$ are primary targets for inactivation by promoter methylation in the acceleration of CML. Transcriptional silencing of the $p16^{INK4A}$ and $p14^{ARF}$ genes may be important in the conversion of CML from the CP to the AP.

Keywords: Chronic myeloid leukaemia; Acceleration; $p16^{INK4A}$; $p14^{ARF}$; Promoter methylation

1. Introduction

Chronic myeloid leukaemia (CML) is a clonal disorder of pluripotent haematopoietic stem cells with a biphasic or triphasic clinical course [1]. The term accelerated phase (AP) is used for patients who have been undergoing treatment for some time and have shown a variety of signs of disease progression, but who do not meet the criteria for blastic disease [2]. The karyotypic hallmark of CML is the Philadelphia (Ph) chromosome, which is characterised at the molecular level by a reciprocal translocation between chromosomes 9 and 22, resulting in the formation of a *bcr-abl* transcript and protein [3]. Although activation of c-*abl* is thought to be important in the pathogenesis of the chronic phase (CP) of CML, further genetic events may be important in the evolution of CML to the AP and blast crisis (BC).

The human *INK4A/ARF* locus on chromosome 9p21 uniquely encodes two distinct tumour suppressors by using separate promoters and alternative reading frames. The two distinct proteins encoded by the *INK4A/ARF* locus are specified by translating the common second exon in alternative reading frames. The cyclin-dependent kinase inhibitor $p16^{INK4A}$ is specified by an RNA comprising exons 1α, 2 and 3 [4]. The alternative product, designated $p14^{ARF}$ for 'alternative reading frame', is encoded by the slightly smaller β transcript that comprises exons 1β, 2 and 3 [5]. The $p16^{INK4A}$ gene is inactivated by mutations, homozygous deletions, or gene methylation in many tumours of diverse origin [6]. Mutations of the *INK4A/ARF* locus affect $p16^{INK4A}$ exclusively or together with $p14^{ARF}$, whereas homozygous deletions at the exon 2 at the *INK4A/ARF* locus affect both $p16^{INK4A}$ and $p14^{ARF}$ [6].

* Corresponding author. Tel.: +36-52-417-565; fax: +36-52-417-565.

E-mail address: mikro@jaguar.dote.hu (F.D. Tóth).

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$p14^{ARF}$ expression is under the control of its own promoter that can be silenced by methylation of CpG islands [7].

The role of $p16^{INK4A}$ and $p14^{ARF}$ gene alterations in the progression of CML is largely unknown. No deletions of $p16^{INK4A}$ were found in the CP of CML [8–10], whereas homozygous deletions of $p16^{INK4A}$ exons were detected in lymphoid BC, but not in myeloid BC [9,10]. Mutations in $p16^{INK4A}$ were reported [11] one out of 4 CML cases who had BC, but the lineage type was not mentioned. In the present work, we studied DNA alterations and the methylation status of $p16^{INK4A}$ and $p14^{ARF}$ genes in 30 patients with Ph chromosome-positive CML. All these patients were studied both at diagnosis and at the onset of AP to determine the possible role of alterations of the $p16^{INK4A}$ and $p14^{ARF}$ genes in the transition of CML from CP to AP.

2. Patients and methods

2.1. Cell specimens

Peripheral blood samples were obtained from leukaemia patients and normal, healthy individuals. The phase of CML was determined by standard clinical and haematological criteria [2]. The CP specimens were collected at presentation, prior to the initiation of treatment. The AP specimens were from patients who had received treatment during the CP of their disease, but had received no treatment after the onset of the AP prior to collection of the samples used in this study. A simple one-step method for purification of human granulocytes, based on the use of a discontinuous gradient of Percoll, was used [12]. The granulocyte populations were represented by mature granulocytes from normal donors, mature and immature granulocytic cells from the CML patients. The purity of the granulocytic elements was higher than 97%.

2.2. Therapy

CML patients were treated according to standard therapeutic protocols as described elsewhere in Ref. [13]. In CP disease, hydroxyurea (HU) was used to reduce the white blood cell number below 20×10^9/l prior to interferon-α (IFN) therapy. Patients with AP disease received an increased dose of IFN combined with low-dose cytosine arabinoside (ara-C). To improve the response rate, combination therapy was completed with HU in some patients. Uniform criteria for haematological and cytogenetic responses were used [13].

2.3. PCR-single strand conformation polymorphism (SSCP) analysis

The $p16^{INK4A}$ and $p14^{ARF}$ genes were analysed for deletions by polymerase chain reaction (PCR)-SSCP analyses. DNA was isolated using a QIAGEN (Valencia, CA, USA) blood kit. Primer sequences for $p16^{INK4A}$ exons 1 and 3 have been described elsewhere in Refs. [14,15]. Primers used in the analysis of $p14^{ARF}$ exon 1 and $p16^{INK4A}$ exon 2 were designed by ourselves from published sequences (Blast Database). The oligonucleotide primers used for amplification of $p14^{ARF}$ exon 1β and $p16^{INK4A}$ exons 1α, 2 and 3 are shown in Table 1. PCR was performed as previously described in Ref. [14]. For SSCP analysis, PCR products were heat-denaturated, treated with formamide and loaded into a neutral 7.5% polyacrylamide gel. Electrophoresis was done for 4 h at 300 V at 4 °C. Finally, bands were visualised by silver nitrate staining.

2.4. Automatic sequencing of $p14^{ARF}$ exon 1β and $p16^{INK4A}$ exons 1α, 2 and 3

The sequences of exons were analysed in each CML sample. Exons were amplified, processed and sequenced using an ABI Prism Big Dye Terminator Ready Reaction

Table 1
Primers used for PCR-SSCP and MSP analyses

Application and specificity	Forward primer (5′→3′)	Reverse primer (5′→3′)	Product size (bp)	Annealing temperature (°C)
PCR-SSCP				
p16 exon 1	GGAGGAAGAAAGAGGAGGG	ACTTCGTCCTCCAGAGTCG	316	60
p16 exon 2	GCTCTGACCATTCTGTTCTC	CTCAGATCATCAGTCCTCAC	355	60
p16 exon 3	GTAGGGACGGCAAGAGA	ACCTTCGGTGACTGATG	159	57
p14 exon 1	GCTCAGAGCCGTTCCGAGAT	TCTCCTCCTCCTCCTAGCCT	368	55
MSP				
p16 promoter M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150	63
p16 promoter U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151	64
p14 promoter M	GTGTTAAAGGGCGGCGTAGC	AAAACCCTCACTCGCGACGA	122	62
p14 promoter U	TTTTTGGTGTTAAAGGGTGGTGTAGT	CACAAAAACCCTCACTCACAACAA	132	60

M, specific for methylated bisulphite-modified DNA; U, specific for unmethylated bisulphite-modified DNA; PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism; MSP, methylation-specific PCR; bp, base pairs.

Kit and an ABI Prism 310 Automatic Sequencer (Applied Biosystem).

2.5. Methylation analysis of $p16^{INK4A}$ and $p14^{ARF}$

The methylation status of the $p16^{INK4A}$ and $p14^{ARF}$ promoters was determined by methylation-specific PCR (MSP) [16]. The oligonucleotide primers used for amplification of $p16^{INK4A}$ promoter/exon 1α [16] and $p14^{ARF}$ promoter/exon 1β [17] are given in Table 1.

2.6. Cytogenetic study

The karyotype of leukaemia cells was investigated by using standard cytogenetic procedures. Chromosomes were identified using G and Q banding techniques.

3. Results

3.1. Haematological and cytogenetic characteristics of patients

The laboratory parameters of the 30 patients with CML are summarised in Table 2. All patients had significant granulocytosis. At the onset of AP, blast cells occasionally appeared in the blood. Other features associated with AP disease included the presence of a high percentage of basophils (cases 2, 8, 11, 15, 16, 19, 24, 27 and 30), thrombocytosis (cases 4, 9, 13, 14, 18, 22, 24 and 25) and anaemia (cases 2, 3, 6, 8, 10, 12, 14, 15, 18, 20, 22, 23, 27 and 30).

Apart from the presence of the Ph chromosome, the karyotype was normal in all patients at the time of first examination. At the onset of the AP of the disease, further chromosomal abnormalities, in addition to the Ph, were demonstrated in 14 of 30 patients. Loss of chromosome 13 or loss of the long arm of chromosome 13 was found in 3 cases. Loss of chromosome 17, the presence of an isochromosome of the long arm of chromosome 17 or shortening of the short arm of chromosome 17 was observed in 5 cases. A second Ph was found in 2 cases. The other chromosomal aberrations included trisomy 8, 19 or 21, loss of chromosomes 5, 7, 18, 20 or 21, and markers 2q− and 16p−. In 11 patients, more than one chromosomal abnormalities was found.

3.2. Deletion analyses of the $p16^{INK4A}$ and $p14^{ARF}$ genes

PCR-SSCP analysis using DNA from 30 patients with CP or AP of CML revealed that none of the patients had homozygous or heterozygous deletions of the $p16^{INK4A}$ or $p14^{ARF}$ genes in their tumour cells (data not shown).

Table 2
Haematological and cytogenetic data of patients

Case no.	Phase of disease	Peripheral blood parameters					Chromosome abnormalities in addition to the Ph
		WBC (10^9/l)	Blast (%)	Ba (%)	Plt (10^9/l)	Hb (g/l)	
1	CP	29	0	1	210	116	
	AP	48	0	2	470	104	
2	CP	32	0	2	230	123	
	AP	98	7	8	258	66	
3	CP	37	0	1	200	110	
	AP	77	1	1	230	85	
4	CP	64	0	2	280	144	
	AP	93	2	2	1040	133	+8, −21
5	CP	61	0	1	243	124	
	AP	103	11	1	290	101	
6	CP	29	0	1	258	113	
	AP	78	0	2	398	90	
7	CP	47	1	0	260	145	
	AP	68	5	4	254	152	
8	CP	86	0	2	230	124	
	AP	70	4	14	240	98	
9	CP	33	0	2	260	129	
	AP	150	5	2	610	108	+8, +19
10	CP	64	0	1	243	148	
	AP	172	2	1	290	84	
11	CP	34	0	2	294	146	
	AP	98	0	6	276	116	
12	CP	28	0	1	232	136	
	AP	45	7	1	220	64	5p+
13	CP	45	0	1	280	152	
	AP	61	1	1	860	136	
14	CP	78	0	2	300	148	
	AP	86	11	2	1215	70	
15	CP	54	0	1	233	139	
	AP	167	4	15	264	94	−13, 17p−
16	CP	86	0	1	241	154	
	AP	70	0	6	220	143	−13q, +19
17	CP	38	1	1	260	124	
	AP	74	1	2	290	112	+8, −13q
18	CP	39	0	1	258	113	
	AP	327	2	2	1846	90	i (17q), −21
19	CP	49	0	1	243	138	
	AP	89	3	7	286	136	−17, +21
20	CP	64	1	1	234	152	
	AP	247	5	1	210	91	17p−, i (17q)
21	CP	45	2	1	230	124	
	AP	72	6	1	280	127	+8, −20, +Ph
22	CP	38	1	1	245	143	
	AP	57	1	3	740	78	+Ph
23	CP	49	1	1	244	134	
	AP	168	2	2	234	91	−7, −18
24	CP	98	0	2	250	152	
	AP	99	0	7	852	138	−5
25	CP	42	0	1	210	128	
	AP	54	8	1	660	119	2q−, +8, 16p−
26	CP	49	0	1	210	145	
	AP	67	9	4	280	115	
27	CP	109	0	2	246	129	
	AP	126	0	14	230	61	
28	CP	52	0	1	276	139	
	AP	83	5	2	290	119	
29	CP	39	1	1	310	143	
	AP	81	2	4	340	127	
30	CP	38	0	1	258	129	
	AP	65	2	6	267	88	

WBC, white blood cells; Ba, basophils; Plt, platelets; Hb, haemoglobin; ph, Philadelphia chromosome; CP, chronic phase; AP, accelerated phase.

3.3. Mutations in $p16^{INK4A}$ and $p14^{ARF}$ genes

DNA sequencing analyses detected mutations in 17 AP samples (Table 3), but in none of the CP samples. Peaks in the sequence chromatograms showed that the mutation was heterozygous in each case.

We found point mutations in exon 1 of *p16*^{INK4A} in 10 patients. A G→A transition at codon 12 was present in 1 patient, but did not result in an amino acid change. As the nucleotide substitution was not seen in the CP of CML, it was considered to be a silent mutation rather than a polymorphism. A specimen had a point mutation (GCG→GTG) in codon 26, resulting in an Ala to Val amino acid substitution. In 6 AP samples, codon 35 was changed from AGT to ATT resulting in an amino acid change from Ser to Ile. The remaining two patients had a nonsense mutation of GAG to TAG at codon 18.

Mutations in exon 2 of *p16*^{INK4A} were detected in 11 patients. In five samples, a silent change from Glu (GAG) to Glu (GAA) was observed. Missense mutations in codons 61, 105, 123 or 130 were detected in 6 cases. Alterations of two bases at codons 61 (GAG→CAG) and 105 (CTG→ATG) resulted in amino changes from Glu to Gln and Leu to Met, respectively. In 2 cases, codon 123 was changed from CGC to CAC resulting in a substitution of Arg with His. In two samples, the mutation was at codon 130 (AGA→ATA), resulting in a change of Arg to Ile.

Because *p16*^{INK4A} and *p14*^{ARF} share exon 2 with different open reading frames, a silent mutation of GAG to GAA at codon 80 of *p16*^{INK4A} resulted in a missense mutation of GGG to AGG at codon 103 of *p14*^{ARF}. Missense mutations identified in codons 61 and 105 of *p16*^{INK4A} resulted in missense mutations at codons 83 and 127 of *p14*^{ARF}, respectively.

Mutations in exon 1 of *p14*^{ARF} were found in 3 patients. In 2 cases, a missense AGG to AAG mutation (Arg→Lys) was observed in codon 21. In a third AP sample, codon 34 was changed from TGG to AGG resulting in a substitution of Trp with Arg.

No mutations were detected in exon 3 in any of the cases.

Table 3
Alterations of the *p16*^{INK4A} and *p14*^{ARF} genes in the accelerated phase (AP) of CML

Case no.	*p16*^{INK4a}					*p14*^{ARF}				
	Mutation				Methylation status	Mutation				Methylation status
	Exon	Codon	Nucleotide change	Coding effect		Exon	Codon	Nucleotide change	Coding effect	
1	2	80	GAG→GAA	Glu→Glu	M	2	103	GGG→AGG	Gly→Arg	M
2					M	1	21	AGG→AAG	Arg→Lys	M
	2	80	GAG→GAA	Glu→Glu		2	103	GGG→AGG	Gly→Arg	
3	2	130	AGA→ATA	Arg→Ile	M					M
4	1	18	GAG→TAG	Glu→stop	M					M
	2	123	CGC→CAC	Arg→His						
5	1	26	GCG→GTG	Ala→Val	M					M
	2	61	GAG→CAG	Glu→Gln		2	83	GGA→GCA	Gly→Ala	
6					M	1	34	TGG→AGG	Trp→Arg	M
	2	105	CTG→ATG	Leu→Met		2	127	TCT→TAT	Ser→Tyr	
7					M	1	21	AGG→AAG	Arg→Lys	M
	2	80	GAG→GAA	Glu→Glu		2	103	GGG→AGG	Gly→Arg	
8					M					M
9					M					M
10					M					M
11	1	18	GAG→TAG	Glu→stop	U					M
12					U					M
13					M					U
14					M					U
15	1	35	AGT→ATT	Ser→Ile	U					U
16	2	123	CGC→CAC	Arg→His	U					U
18	1	35	AGT→ATT	Ser→Ile	U					U
19	2	80	GAG→GAA	Glu→Glu	U	2	103	GGG→AGG	Gly→Arg	U
22	1	12	GCG→GCA	Ala→Ala	U					U
	2	130	AGA→ATA	Arg→Ile						
23	1	35	AGT→ATT	Ser→Ile	U					U
25	1	35	AGT→ATT	Ser→Ile	U					U
27	1	35	AGT→ATT	Ser→Ile	U					U
	2	80	GAG→GAA	Glu→Glu		2	103	GGG→AGG	Gly→Arg	
29	1	35	AGT→ATT	Ser→Ile	U					U

M, methylated; U, unmethylated; CML, chronic myeloid leukaemia.

3.4. *Methylation status of the* $p16^{INK4A}$ *and* $p14^{ARF}$ *genes*

Thirty CP and AP samples were analysed for $p16^{INK4A}$ and $p14^{ARF}$ promoter methylation by using MSP. There was no detectable methylation of the $p16^{INK4A}$ and $p14^{ARF}$ genes in any of the samples from the CP of CML. We found a similar frequency of promoter methylation in the AP of CML for both genes (Table 3). Representative analyses for $p16^{INK4A}$ and $p14^{ARF}$ in the AP of CML are shown in Fig. 1. In each case, in addition to the presence of a methylation-specific PCR signal, a weak PCR signal corresponding to the unmethylated DNA sequence could also be detected from the same sample. Methylation of the $p16^{INK4A}$ promoter was detected in 12 of the 30 AP samples (cases 1–10, 13 and 14). Ten AP samples with $p16^{INK4A}$ methylation also had a methylated $p14^{ARF}$ gene (cases 1–10). Methylation of the $p14^{ARF}$ promoter alone was found in 2 AP samples (cases 11 and 12).

Simultaneous appearance of point mutations and the methylated status was observed in eight AP samples (cases 1–7 and 11), whereas 6 AP samples (cases 8–10, 12–14) showed aberrant methylation without sequence alterations.

3.5. *Response to therapy during the accelerated phase of CML*

Table 4 shows the haematological and cytogenetic responses to therapy in the AP of CML. Of the 30 patients, 10 had complete haematological remissions and partial remissions were observed in 14 patients. 6 patients were refractory to the therapy. Of the 10 patients with complete haematological remission, 8 had suppression of Ph-positive metaphases: 3 partial cytogenetic remissions and 5 minor cytogenetic responses. Except for 1 case (No. 15), loss of response to therapy was associated with aberrant methylation of both the $p16^{INK4A}$ and $p14^{ARF}$ genes, with or without sequence alterations (cases 2, 5, 8–10). None of the patients with $p16^{INK4A}$ or $p14^{ARF}$ promoter methylation obtained complete haematological remission upon therapy. In contrast, complete haematological remission was achieved in 4 patients with mutationally altered $p16^{INK4A}$ (cases 16, 22, 25 and 29).

4. Discussion

Studies on haematopoietic malignancies have demonstrated two major types of $p16^{INK4A}$ alterations, the prevalence of which varies with the type of leukaemias and lymphomas. Large homozygous deletions of $p16^{INK4A}$ are predominantly associated with malignancies of lymphocytes, foremost among them is acute lymphoblastic leukaemia of the T cell lineage followed closely by B cell precursor acute lymphoblastic leukaemia and adult T cell leukaemia-lymphoma [18,19]. Homozygous deletions of $p16^{INK4A}$ have also been found in lymphoid BC of CML [9,10], which behaves like an acute lymphoblastic leukaemia. The tumour cells of some haematopoietic disorders that are not afflicted by $p16^{INK4A}$ deletions display the second major option of $p16^{INK4A}$ gene inactivation, namely promoter methylation, which is seen at high levels in Burkitt's

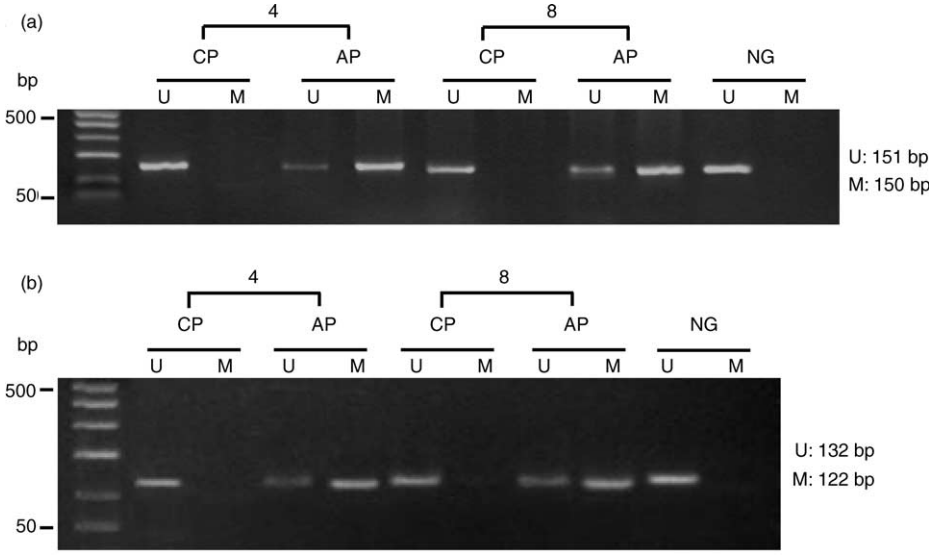

Fig. 1. Representative samples of methylation-specific PCR results for the $p16^{INK4A}$ (a) and $p14^{ARF}$ (b) genes. Chronic phase (CP) and accelerated phase (AP) samples from 2 chronic myeloid leukaemia (CML) cases designated below the patient numbers are shown. Patient numbers are identical to those listed in Tables 2 and 3. Lanes U and M correspond to the unmethylated and methylated reactions, respectively. Normal granulocytes (NG) from a healthy person served as a control.

Table 4
Response to therapy in accelerated phase (AP) of CML

Case no.	Therapy in		Response to therapy in AP	
	CP	AP	Haematological remission	Cytogenetic response
1	IFN	HU + IFN + Ara-C	Partial	No
2	HU + IFN	HU + IFN + Ara-C	No	No
3	HU + IFN	HU + IFN + Ara-C	Partial	No
4	HU + IFN	HU + IFN + Ara-C	Partial	No
5	HU + IFN	HU + IFN + Ara-C	No	No
6	IFN	HU + IFN + Ara-C	Partial	No
7	HU + IFN	HU + IFN + Ara-C	Partial	No
8	HU + IFN	HU + IFN + Ara-C	No	No
9	IFN	HU + IFN + Ara-C	No	No
10	HU + IFN	HU + IFN + Ara-C	No	No
11	HU + IFN	HU + IFN + Ara-C	Partial	No
12	IFN	HU + IFN + Ara-C	Partial	No
13	HU + IFN	HU + IFN + Ara-C	Partial	No
14	HU + IFN	HU + IFN + Ara-C	Partial	No
15	HU + IFN	HU + IFN + Ara-C	No	No
16	HU + IFN	IFN + Ara-C	Complete	Minor
17	HU + IFN	IFN + Ara-C	Complete	Partial
18	HU + IFN	HU + IFN + Ara-C	Partial	No
19	HU + IFN	HU + IFN + Ara-C	Partial	No
20	HU + IFN	HU + IFN + Ara-C	Partial	No
21	HU + IFN	IFN + Ara-C	Complete	Minor
22	HU + IFN	IFN + Ara-C	Complete	Minor
23	HU + IFN	HU + IFN + Ara-C	Partial	No
24	HU + IFN	IFN + Ara-C	Complete	Minor
25	HU + IFN	IFN + Ara-C	Complete	No
26	HU + IFN	IFN + Ara-C	Complete	No
27	HU + IFN	HU + IFN + Ara-C	Partial	No
28	HU + IFN	IFN + Ara-C	Complete	Minor
29	HU + IFN	IFN + Ara-C	Complete	Partial
30	HU + IFN	IFN + Ara-C	Complete	Partial

HU, hydroxyurea; IFN, interferon-α; Ara-C, cytosine arabinoside.

lymphoma, Hodgkin's lymphoma and multiple myeloma [20]. In contrast to $p16^{INK4A}$, there is little known regarding the role of $p14^{ARF}$ in haematopoietic malignancies. Many homozygous deletions at the *INK4A/ARF* locus simultaneously disrupt both $p16^{INK4A}$ and $p14^{ARF}$ genes in T cell acute lymphoblastic leukaemia [21]. Repression of the $p14^{ARF}$ promoter by the AML1-ETO fusion protein has been demonstrated in acute myeloid leukaemia, representing a distinct mechanism for tumour suppressor inactivation [22].

The present study was designed to determine both the frequency and mechanism of $p16^{INK4A}$ and $p14^{ARF}$ gene alterations leading to acceleration of CML. No homozygous or heterozygous deletions of the $p16^{INK4A}$ and $p14^{ARF}$ genes were found in any sample. Our failure to detect deletions in tumour cells from the CP or AP of CML is compatible with results from other studies [10,11,19]. In AP samples, we detected relatively large numbers of mutations at the *INK4A/ARF* locus which, to our knowledge, had not been previously reported. Since no mutations were found in CP samples prior to the initiation of treatment, it cannot be excluded that these mutations were induced mostly by chemotherapy given in the CP of CML. All mutations were heterozygous. Exon 1α mutations were found in 10 cases. Seven of the 11 $p16^{INK4A}$ mutations in exon 2 simultaneously affected $p14^{ARF}$. We could find exon 1β mutations in 3 cases. None of the 30 AP samples had mutations in exon 3. Given the small number of mutations detected in exon 1β, the $p14^{ARF}$ gene seems mostly to be an indirect target for inactivation due to the different reading frames in exon 2 of both genes. Missense mutations were observed more frequently than silent or nonsense mutations. Two mutational hot spots of the *INK4A/ARF* locus have been detected. One of them was found at codon 35 (Ser to Ile) in exon 1 of $p16^{INK4A}$. The other mutational hot spot was located to codon 80 in exon 2 of $p16^{INK4A}$. A silent mutation identified in codon 80 of $p16^{INK4A}$ resulted in an amino acid change at codon 103 of $p14^{ARF}$. These mutational hot spots of the $p16^{INK4A}$ gene are different from those detected in other tumours [23]. Our study is the first to find methylation defects in the promoter region of $p16^{INK4A}$ and $p14^{ARF}$ in the AP of CML. Aberrant methylation of

the *p16*^{INK4A} or *p14*^{ARF} promoters was found in nearly half of the cases. In our set of AP samples, the most common situation was the simultaneous methylation of both promoters. Our methylation results with regard to the *p16*^{INK4A} promoter have both similarities to and differences from those reported in one study, which failed to detect aberrant methylation of the *p16*^{INK4A} promoter in both the CP and AP of CML [24]. The reason for the lack of *p16*^{INK4A} promoter methylation in the above study may be due to the small number of AP samples.

The *p16*^{INK4A} and *p14*^{ARF} proteins exert their growth control activity via the Rb and p53 pathways, respectively. The *p16*^{INK4A} protein induces a G_1 cell-cycle arrest by binding to and inhibiting the activity of cyclin-D-dependent kinases, CDK4 and CDK6, thereby maintaining the Rb protein in its growth suppressive, hypophosphorylated state [4]. The *p14*^{ARF} protein restrains cell growth by abrogating MDM2 inhibition of p53 activity, resulting in a distinctive cell-cycle arrest in both the G_1 and G_2/M phases [5]. The high level of methylated DNA and low level of unmethylated DNA in the same AP samples strongly suggests that *p16*^{INK4A} and *p14*^{ARF} promoters were fully methylated in CML cells and the unmethylated DNA might come from normal granulocytes, which cannot be separated from the CML cells. It has been shown that aberrant promoter methylation correlates with a lack of *p16*^{INK4A} and *p14*^{ARF} protein expression and results in collapse of the Rb and p53 growth control pathways, respectively [25,26]. Thus, the tumour cells with inactivated *p16*^{INK4A} and *p14*^{ARF} genes may have a selective growth advantage. Results of therapy in the AP of CML suggest that epigenetic silencing of *p16*^{INK4A} and *p14*^{ARF} expression is a property of tumour progression and might predict a poor clinical outcome. In contrast to the biallelic methylation, the significance of heterozygous mutations without loss of the other allele is unclear in the acceleration of CML.

Abnormalities in chromosomes 13 and 17 that were found in 6 patients with an AP of CML suggest that structural alterations of the *Rb* and *p53* genes may also be involved in the acceleration of CML [27]. Several other tumour suppressor genes [28] have been mapped to specific chromosomal locations: *PMS*-1 was mapped to chromosome 2; *APL* to chromosome 5; *PMS*-2 to chromosome 7; *TSC*-2 to chromosome 16; and *DCC* to chromosome 18. We observed karyotypic abnormalities 2q−, −5, −7, 16p− and −18 in several AP samples. Although the significance of these changes is not known, the loss of *APL*, *DCC*, *PMS*-1, *PMS*-2 and *TSC*-2 might also be of pathogenic importance in the transition of CML from its CP to acceleration. A second Ph chromosome found in 2 patients with an AP of CML may also provide a growth advantage for leukaemia cells, although the appearance of a plus Ph chromosome is a relatively rare event in the AP of CML [29]. Other genetic alterations, involving an increase in oncogene expression or expression of additional oncogenes [30], may also accelerate disease progression.

In conclusion, our results indicate that *p16*^{INK4A} and *p14*^{ARF} are primary targets for inactivation in the acceleration of CML, and aberrant promoter methylation is the main event underlining *p16*^{INK4A} and *p14*^{ARF} inactivation.

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References

1. Kantarjian HM, Deisseroth A, Kurzrock R, Estrov Z, Talpaz M. Chronic myelogenous leukemia. A concise update. *Blood* 1993, **82**, 691–703.
2. Sokal JE, Baccarini M, Russo D, Tura S. Staging and prognosis in chronic myelogenous leukaemia. *Semin Hematol* 1988, **25**, 49–61.
3. Konopka JB, Watanabe SM, Witte ON. An alteration of human c-*abl* protein in K562 leukemia cells unmasks associated tyrosine kinase activity. *Cell* 1884, **37**, 1035–1039.
4. Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 1993, **366**, 704–707.
5. Stott FJ, Bates S, James MC, *et al*. The alternative product from the human CDKN2A locus, p14^{ARF}, participates in a regulatory feedback loop with p53 and MDM2. *EMBO J* 1998, **17**, 5001–5014.
6. Ruas M, Peters G. The p16^{INK4A} /CDKN2A tumor suppressor and its relatives. *Biochem Biophys Acta* 1998, **1378**, F115–F177.
7. Esteller M, Tortola S, Toyota M, *et al*. Hypermethylation-associated inactivation of p14^{ARF} is independent of p16^{INK4a} methylation and p53 mutational status. *Cancer Res* 2000, **60**, 129–133.
8. Haidar MA, Cao XB, Manshouri T, *et al*. p16^{INK4A} and p15^{INK4B} gene deletions in primary leukemias. *Blood* 1995, **86**, 311–315.
9. Serra A, Gottardi E, Della Ragione F, Saglio G, Iolascon A. Involvement of the cyclin-dependent kinase-4 inhibitor (CDNK2) gene in the pathogenesis of lymphoid blast crisis of chronic myelogenous leukemia. *Br J Haematol* 1995, **91**, 625–629.
10. Sill H, Goldman JM, Cross NCP. Homozygous deletions of the p16 tumor-suppressor gene are associated with lymphoid transformation of chronic myeloid leukemia. *Blood* 1995, **85**, 2013–2016.
11. Güran S, Bahce M, Beyan C, Korkmaz K, Yalcin A. P53, p15^{INK4B}, p16^{INK4A}, and p57^{KIP2} mutations during the progression of chronic myeloid leukemia. *Hematologia* 1998, **29**, 181–193.
12. Hjorth R, Jonsson AK, Vretblad P. A rapid method for purification of human granulocytes using Percoll. A comparison with dextran sedimentation. *J Immunol Methods* 1981, **43**, 95–101.
13. Kantarjian HM, Giles FJ, O'Brien SM, Talpaz M. Clinical course and therapy of chronic myelogenous leukaemia with interferon-alpha and chemotherapy. *Hematol Oncol Clin North Am* 1998, **12**, 31–80.
14. Chen TC, Hsieh LL, Kuo TT, *et al*. p16^{INK4} gene mutation and allelic loss of chromosome 9p21-22 in Taiwanese hepatocellular carcinoma. *Anticancer Res* 2000, **20**, 1621–1626.

15. Baur AS, Shaw P, Burri N, Delacrétaz F, Bosman FT, Chaubert P. Frequent methylation silencing of $p15^{INK4b}$ (MTS2) and $p16^{INK4a}$ (MTS1) in B-cell and T-cell lymphomas. *Blood* 1999, **94**, 1773–1781.
16. Herman JG, Graff JR, Myöhänen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996, **93**, 9821–9826.
17. Zöchbauer-Müller S, Fong KM, Virmani AK, Geradts J, Gazdar AF, Minna JD. Aberrant promoter methylation of multiple genes in non-small cell lung cancers. *Cancer Res* 2001, **61**, 249–255.
18. Hirama T, Koeffler HP. Role of the cyclin-dependent kinase inhibitors in the development of cancer. *Blood* 1995, **86**, 841–854.
19. Ogawa S, Hangaishi A, Miyawaki S, *et al*. Loss of the cyclin-dependent kinase 4-inhibitor (p16, MTS1) gene is frequent in and highly specific to lymphoid tumors in primary human hematopoietic malignancies. *Blood* 1995, **86**, 1548–1556.
20. Krug U, Ganser A, Koeffler HP. Tumor suppressor genes in normal and malignant hematopoiesis. *Oncogene* 2002, **21**, 3475–3495.
21. Gardie B, Cayuela JM, Martini S, Sigaux F. Genomic alterations of the $p19^{ARF}$ encoding exons in T-cell acute lymphoblastic leukemia. *Blood* 1998, **21**, 1016–1020.
22. Linggi B, Müller-Tidow C, van de Locht L, *et al*. The t (8, 21) fusion protein, AML1-ETO, specifically represses the transcription of the $p14^{ARF}$ tumor suppressor in acute myeloid leukemia. *Nat Med* 2002, **8**, 743–750.
23. Caldas C, Hahn SA, Costa LT, *et al*. Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. *Nat Med* 1994, **8**, 27–32.
24. Nguyen C, Liang G, Nguyen TT, *et al*. Susceptibility of non-promoter CpG islands to *de novo* methylation in normal and neoplastic cells. *J Natl Cancer Inst* 2001, **93**, 1465–1472.
25. Suh SI, Pyun HY, Cho JW, *et al*. 5-Aza-2′-deoxycytidine leads to down-regulation of aberrant $p16^{INK4A}$ RNA transcripts and restores the functional retinoblastoma protein pathway in hepatocellular carcinoma cell lines. *Cancer Lett* 2000, **160**, 81–88.
26. Esteller M, Cordon-Cardo C, Corn PG, *et al*. $p14^{ARF}$ silencing by promoter hypermethylation mediates abnormal intracellular localization of MDM2. *Cancer Res* 2001, **61**, 2816–2821.
27. Beck Z, Kiss A, Tóth FD, *et al*. Alterations of *P53* and *RB* genes and the evolution of the accelerated phase of chronic myeloid leukemia. *Leuk Lymphoma* 2000, **38**, 587–597.
28. Hesketh R. *The Oncogene Facts Book*. San Diego, Academic Press, 1995, 295–336.
29. Swolin B, Weinfeld A, Westin J, Waldenström J, Magnusson B. Karyotypic evolution in Ph-positive chronic myeloid leukemia in relation to management and disease progression. *Cancer Genet Cytogenet* 1985, **18**, 65–79.
30. Beck Z, Bácsi A, Kovács E, *et al*. Changes in oncogene expression implicated in evolution of chronic granulocytic leukemia from its chronic phase to acceleration. *Leuk Lymphoma* 1998, **30**, 293–306.