Involvement of INK4 Family Cyclin-dependent-kinase Inhibitor Genes in Hematological Malignancies

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ABSTRACT. Recent studies of the biochemistry of cell cycle progression have led to the discovery of a group of small proteins called cyclin-dependent-kinase inhibitors (CDK-I), CDK-Is. These proteins interact with and inhibit the activity of cyclin/cyclin-dependent-kinases complexes and act as a brake on cell cycle progression. Some of these small proteins such as *p16* and *p15* genes appear to behave as tumor suppressor genes in a variety of neoplasms, including hematological malignancies. Numerous studies have been published regarding the inactivation of these genes in human malignant neoplasms. In hematological malignancies, homozygous deletion is a frequent mechanism of inactivation of *p16* and *p15* in acute lymphocytic leukemias with immature blasts, particularly in those with T-cell phenotypes. In this manuscript, we have presented the results of investigations performed in the Hematopathology Section of the Laboratory of Pathology, National Cancer Institute and summarized the existing literature concerning the abnormalities *p16* and/or *p15* genes in hematological malignancies and solid tumors. We have also reviewed literature that suggests that hypermethylation of the 5'CpG island of the *p16* gene may also result in transcriptional silencing of the gene and lead to its functional inactivation. The combined data suggests that the CDK-I genes play an important role in the genesis of acute lymphoid leukemias as well as in the genesis of other types of solid tumors, through at least three different mechanisms of gene inactivation. Nonetheless, additional studies are necessary to further define the biologic role of these genes in human cancers, and to elucidate the clinical consequences of alterations of these genes in human cancers.

Key words: cyclin-dependent-kinase — inhibitor — hematological malignancy — solid tumor — tumor suppressor gene — methylation

CYCLIN-DEPENDENT-KINASE INHIBITOR GENES

Recent advances in the molecular biology of cell cycle progression have uncovered a group of small proteins called cyclin-dependent-kinase inhibitors (**CDK-Is**) which interact with cyclin/cyclin-dependent-kinases (**CDKs**) complexes to negatively regulate progression of cells through cell cycle.[1-7] These CDK-Is can be divided into two families, one whose members inhibit cyclin/

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CDK kinase activity through the formation of a ternary cyclin/CDK/CDK-I complex, and a second whose members inhibit cyclin/CDK kinase activity by preventing the formation of the initial cyclin/CDK complex by competitively binding the CDK subunits.[8-13] The former CDK-I family includes *p21*,[14] *p27*,[15,16] and *p57*.[18,19] The latter family consists of *p16*,[20] *p19*^{ARF},[21] *p15*,[22] *p18*,[23] and *p19*.[24,25] This latter family has been designated the INK4 family (inhibitor of CDK4) because its members preferentially bind to CDK4 to inhibit the enzymatic activity of the cyclin D/CDK4 complex.[7,8,10-12]

The members of p21 family bind a wide variety of cyclin/CDK complexes. *p21*[14] (also known as Sdi1,[26,27] WAF1,[28] Cip1,[29] or CAP20,[30] located at chromosome 6p21.2,[28] inhibits the activities of cyclin D/CDK4, cyclin E/CDK2, and cyclin A/CDK2 complexes. *p21* is also a target of the p53 tumor suppressor gene. The *p21* promoter contains a p53 binding site, and *p21* expression is induced by the wild type but not mutant type p53 gene.[31-33] In addition, *p21* can inhibit DNA replication in the absence of cyclin/CDK by binding to PCNA (proliferating cell nuclear antigen) and, thereby, inhibiting the activity of DNA polymerase δ.[34-36] Thus, *p21* appears to function in the response to DNA damage through its ability to interfere with cyclin/CDK activities during the G1 phase of the cell cycle and by blocking DNA synthesis during S phase, allowing cells to repair DNA damage.

p27, also known as Kip1,[16,17] is located on chromosome 12p13.[37-40] A 60 amino acid (AA) segment at the terminal end of *p27* is 44% identical to the corresponding sequences of *p21*. *p27* binds preferentially to already formed cyclin/CDK complexes rather than CDKs alone, and is capable of inhibiting the activity of cyclin D/, E/, A/, and B/CDK complexes.[41-44] This gene was initially identified as a protein bound to the cyclin E/CDK3 complex in TGFβ arrested cells.[45] Surprisingly, it has been recently reported that targeted disruption of the *p27* gene causes enhancement of growth of mice associated with multiorgan hyperplasia, female sterility, giantism, and pituitary tumors. These results indicate that *p27* is critical to the normal functioning of hypothalamus-pituitary-ovarian axis.[46-48]

p57,[18,19] also known as Kip2, contains an amino-terminal CDK inhibitory domain homologous to both *p21* and *p27*, and is located chromosome 11p15.5. *p57* protein binds to CDK2, 3, 4, and 6 and inhibits the kinase activities of cyclin D/CDK4, D/CDK6, E/CDK3, and A/CDK2 complexes.

The members of INK4 family bind to CDK4 or CDK6 but not to cyclins or other known CDKs. All five members of this family, *p16* (INK4a, CDKN2, MTS1) *p19*^{ARF}, *p15* (INK4b, MTS2), *p18* (INK4c), and *p19* (INK4d), are located at chromosome 9p21,[49,50] 9p21,[21] 9p21,[49,50] 1p32,[23] and 19p13,[24,25] respectively. *p15* is located approximately 25 kb centromeric to the *p16* gene. Interestingly, the *p19*^{ARF} coding sequence overlaps that of *p16*, but uses an alternative reading frame and a unique promoter.[21,51] All of these genes, with the exception of *p19*^{ARF} show structural homology to each other. In addition, all can inhibit cyclin D/CDK4 and cyclin D/CDK enzymatic actvities by competing with cyclin D for binding CDK4 or 6.[7,8,10-12]

Recent molecular genetic analyses have implicated *p16* and *p15* as putative tumor suppressor genes. A high frequency of homozygous deletion of both genes, and somatic mutations of *p16* have been reported in a variety of human cancer cell lines and primary tumors. However, in other tumors, somatic

mutations are infrequent, leaving some uncertainty regarding the role of the two genes as tumor suppressors (details will be discussed later). On the other hand, there have been several reports demonstrating that transfection of *p16* into cells containing wild type Rb protein leads to cell cycle arrest and growth inhibition, whereas transfection with tumor-associated mutated *p16* genes do not.[51–55] Moreover, Serrano et al have demonstrated that *p16* deficient mice develop spontaneous tumors at an early age, and are highly sensitive to carcinogenic treatment.[57] In addition, *p16*-deficient primary fibroblasts proliferate rapidly and have a high cloning efficiency. These experiments suggest that *p16* functions to suppress neoplastic growth and may play a role as a tumor suppressor gene *in vivo*.

The *p16* gene contains four exons (E1α, E1β, E2 and E3) that generate two different types of transcripts (named MTS1α and MTS1β), each initiated from distinct promotors associated with E1α, E1β.[21,22,58–60] The MTS1α transcript utilizes E1α, E2 and E3 and encodes *p16* INK4a, whereas the MTS1β transcript utilizes a novel 5' exon (E1β), E2 and E3. Splicing of E1β into E2 generates an alternative reading frame which results in a novel gene encoding a 132 amino acid polypeptide ($p19^{ARF}$) that shows no structual homology to *p16* INK4a.

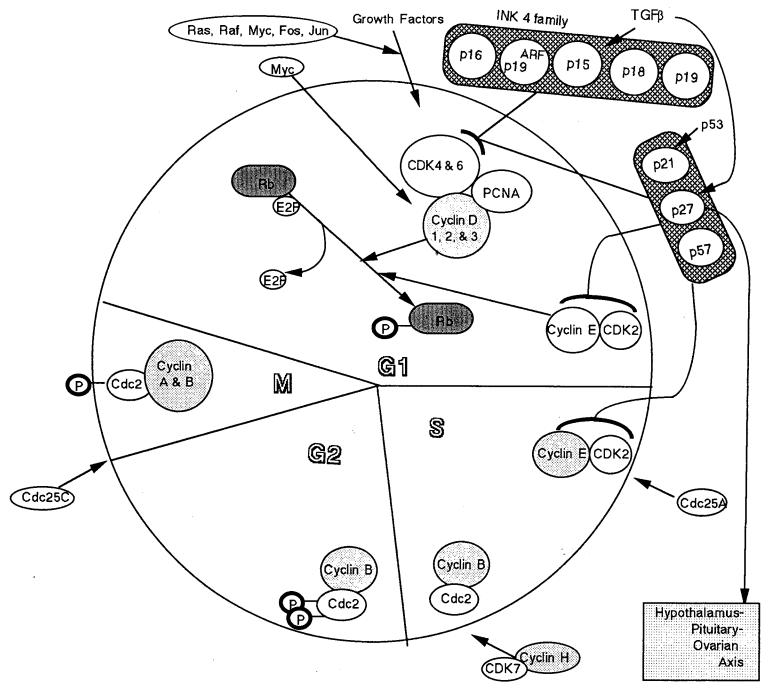

Fig 1. Schematic model of cell cycle progression depicting the interaction among cyclins, cyclin-dependent-kinase (CDK), INK4 family CDK-inhibitors (CDK-I) (*p16*, *p15*, *p18*, and *p19*), p21 family CDK-Is (*p21*, *p27*, *p57*), retinoblastoma gene, other c-oncogenes, and growth factors.

p15 was initially identified as a protein associated with CDK4 in TGFβ-arrested cells and sharing 50% identity with *p16*.[22] *p18* and *p19* were

identified as CDK4 or 6 binding proteins. They also interfere with the formation of the activated cyclin/CDK complexes. *p18* shares 38% amino acid identity with *p16* over a 150 AA-region, and 42% identity with *p15* over a 129 amino acid-region.[23] *p19* shares a similar degree of structural homology to *p15* and *p16*.[24,25]

As summarized in (Fig 1), the CDK-I genes seem to play an important role not only in cell cycle progression but also tumorigenesis in human cancers, including hematological malignancies.

INVOLVEMENT OF INK4 FAMILY CDK-IS IN HEMATOLOGICAL MALIGNANCIES

Deletional and mutational analyses performed in Section of Hematopathology, Laboratory of Pathology, National Cancer Institute[61,62]

Cell lines studied. Fourteen human hematological malignant cell lines were studied. These included 6 B-cell derived cell lines (2 EBV transformed lymphoid cell lines [SB and RPMI-8392], 1 null ALL cell line [NALL], 2 Burkitt's lymphoma cell lines [Raji and Dandi], 1 anaplastic large cell line [SU-DHL-1], 4 T-ALL lines [CEM, Molt4, RPMI-8402, and HSB], and 3 non-lymphoid leukemia cell lines [HL60, U937, and K562].

Cases Studied. A total of 117 lymphoid tumor samples from patients referred to the Hematopathology Section of the Laboratory of Pathology, National Cancer Institute were selected for these studies based on the availability of frozen tissue or previously extracted DNA for molecular analysis. Only leukemias and aggressive lymphomas with high percentages of tumor cells (> 70%) were studied to facilitate deletion interpretation. The number of tumor samples for *p16/p15* studies and *p18* study were follows;

Lineage	Tumor type	Number of cases studied	
		p16/p15	*p18*
T	ALL/LBL	23	18
	CLL	3	4
	PTCL	36	18
	ATL	3	0
B	CLL	37	30
	NHL	10	9
	ALL	5	2

(Abbreviations used are following; ALL, acute lymphoblastic leukemia; LBL, lymphoblastic lymphoma; CLL, chronic lymphocytic leukemia; PTCL, peripheral T-cell lymphoma; ATL, adult T-cell leukemia/lymphoma; NHL, non-Hodgkin's lymphoma)

Southern blot analysis. High molecular weight DNA was extracted from involved frozen tissue samples or cell suspensions using standard methods.

After restriction enzyme digestion with *Eco*RI or *Xba*I, the DNA was size-fractionated by agarose gel electrophoresis transferred onto nylon membranes, and hybridized with the following probes.

Probes. PCR derived probes to exon 2 of *p16*, exon 2 of *p15*, and exon 2 of *p18* for Southern blot analysis were generated with the following primer sets:

p16 exon 2 probe	1p-5; 5'-GCCGTCTGCCCGTGGACCTG-3'
	1p-3; 5'-TGAGCTTTGGAAGCTCTCAG-3'
p15 exon 2 probe	2p-5; 5'-CGATGCCTGGGGTCGTCTGC-3'
	2p-3; 5'-GCAGCACCACCAGCGTG-3'
p18 exon 2 probe	3p-5; 5'-TTACAGACTTTGCTGGAGT-3'
	3p-3; 5'-TTATTGAAGATTTGTGGCT-3'

A genomic probe for *BCL-2 mbr* (courtesy of Dr Stanley Korsmeyer, Howard Hughes Medical Institute, Washington University School of Medicine, St Louis, MO, USA) and/or to vimentin (Oncor, Gaithersburg, MD, USA) were used to control for DNA loading in all Southern blot analysis.

SSCP (single strand conformational polymorphism)-PCR analysis. A single primer set was used for both exon 1α and 3 of *p16*. Four sets of overlapping primers were used to screen exon 2 of *p16*. One of two alternative 5'-primer sets, 3 or 4, was used to screen the central region of *p16*. Four sets were used for *p15*, with one of two alternative primer sets, 8 and 9, used to screen the central region of *p15*. Similarly, three sets of overlapping primer sets were used for *p18*. The intron 1 sequences necessary for primer selection were determined by amplifying intron 1 by an LT-PCR procedure.[62]

p16 exon 1α	1-5; 5'-GGGAGCAGCATGGAGCCG-3'
	1-3; 5'-AGTCGCCCGCCATCCCCT-3'
p16 exon 2	2-5; 5'-GGCTCTGACCATTCTGTTCTC-3'
	2-3; 5'-GTCGTGCACGGGTCGGGTGAG-3'
	3-5; 5'-AGCCCAACTGCGCCGAC-3'
	3-3; 5'-CGCAGGTACCGTGCGACATCG-3' or
	4-5; 5'-GCTGAGGAGCTGGGCCATCGC-3'
	4-3; 5'-CGCAGGTACCGTGCGACATCG-3'
	5-5; 5'-CGATGCCTGGGGCCGTCTGC-3'
	5-3; 5'-TGAGCTTTGGAAGCTCTCAG-3'
p16 exon 3	6-5; 5'-CCGGTAGGGACGGCAAGAGA-3'
	6-3; 5'-CTGTAGGACCCTCGGTGACTGATGA-3'
p15 exon 2	7-5; 5'-GCCGGCATCTCCCATACCTG-3'
	7-3; 5'-ATCATGCACGGGTCGGGTGAG-3'
	8-5; 5'-AGCCCAACTGCGCAGACCCT-3'
	8-3 and 9-3; 5'-GCGGTGGCCCCGCTCCTCGGC-3'
	9-5; 5'-CCCCACGGCGCGGAGCCCAACTG-3'
	10-5; 5'-CGATGCCTGGGGTCGTCTGC-3'
	10-3; 5'-CGCTCCCCGTTGGCAGCCTT-3'
p18 exon 1	11-5; 5'-TGATCGTCAGGACCCTAAA-3'
	11-3; 5'-AAAGCTGTAAATCTAGAAAC-3'

p18 exon 2 12-5 ; 5'-ATATGCACTTGAAGGATTCT-3'
12-3 ; 5'-AGGTTCCCTTCATTATCC-3'
13-5 ; 5'-TTACAGACTTTGCTGGAGT-3'
13-3 ; 5'-TTATTGAAGATTTGTGGCT-3'

PCRs were performed using 100 ng of genomic DNA under standard conditions. Samples were loaded onto a 6% acrylamide gel containing 10% glycerol. Gel electrophoresis were performed at 50-60W for 3-5 hours.

Sequencing strategy. Direst sequencing of PCR generated fragments was performed using the Sequenase PCR Product Sequencing Kit (United States Biochemical Corp, Cleveland, OH), according to the instructions supplied by the manufacture. 5-30 ng of the amplified PCR product was treated with 1 unit of Exonuclease 1 and 2 units of Shrimp Alkaline Phosphatase at 37℃ for 15 minutes to remove excess primers. Following heat inactivation of the added enzymes, 0.2-0.5 pM of the PCR product was annealed with 10 pmol of the appropriate primer, and the sequencing reaction was performed with the use of [^{35}S]dATP. Autoradiographs were developed after 1-7 days of exposure at ambient temperature.

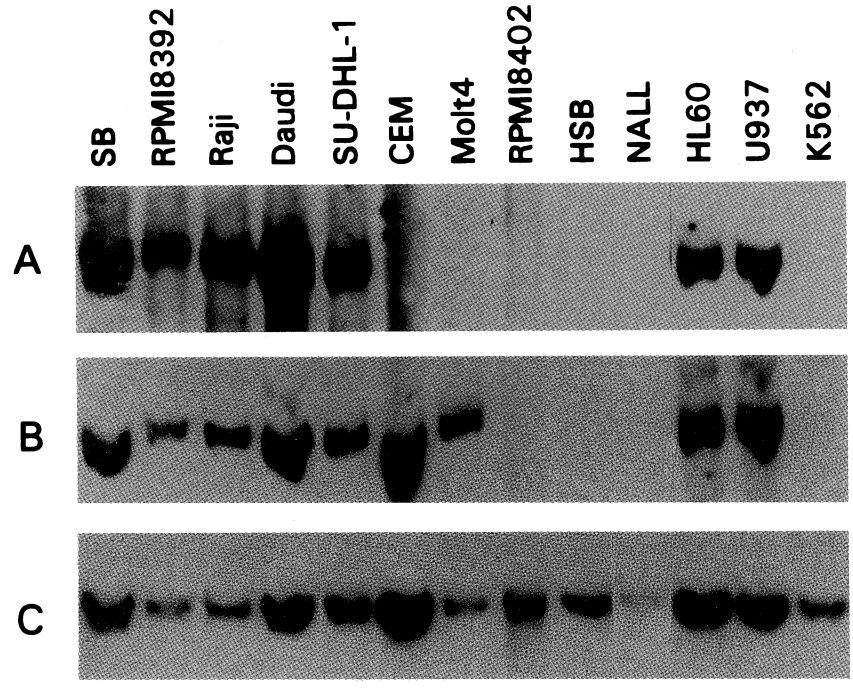

Fig 2. Southern blot analysis of hematopoietic cell lines. High molecular weight DNA from 14 cell lines were digested by *Xba* I and hybridized with PCR-generated probe for *p16* exon 2 (A), *p15* exon 2 (B), and a human *BCL-2 mbr* gene probe to estimate DNA loading (C). Four T-cell derived cell lines, one null ALL cell line, and the erythroleukemia cell line K562 show homozygous deletions of *p16* gene. Homozygous deletions of *p15* are also present in all of the *p16* deleted cell lines except CEM and Molt 4.

Results of Southern blot analysis for deletion of INK4 family CDK-I genes. Homozygous deletions of *p16* were present in all four T-ALL cell lines, 1 null ALL line, and in 1 erythroleukemia cell line (Fig 2). None of the B-cell lines displayed deletions of *p16*. CEM and Molt4 retained *p15*, while the remaining 4 *p16*-deleted cell lines also had homozygous deletions of *p15*. None of cell lines studied showed deletion of *p18* (data not shown).

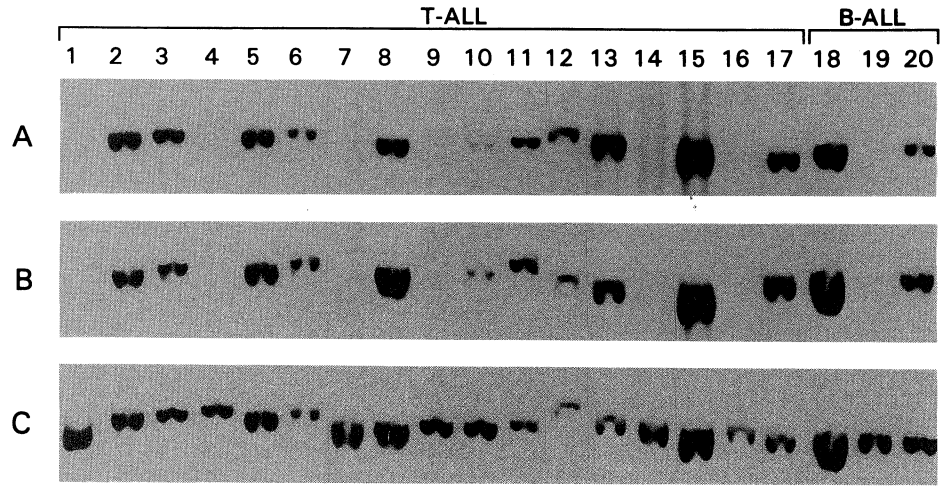

Fig 3. Southern blot analysis of selected T-ALL/LBL and B-ALL cases. High molecular weight DNA from 14 cell lines were digested by *Eco*RI and hybridized with PCR-generated probe for *p16* exon 2 (A), p15 exon 2 (B), and a human *BCL-2 mbr* gene probe to estimate DNA loading (C). All the cases which showed homozygous deletion of the *p16* gene also revealed deletion of the *p15* gene.

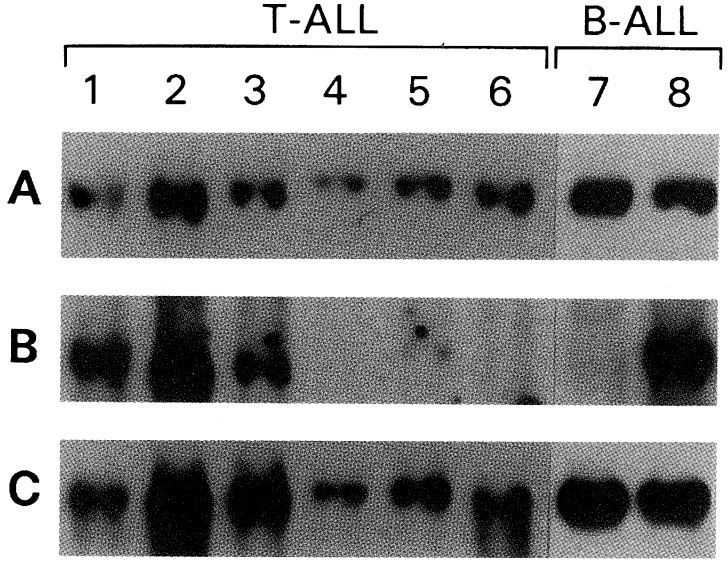

Fig 4. Southern blot analysis of selected T-ALL / LBL and precursor B-ALL cases. High molecular weight DNA from 14 cell lines were digested with *Eco*RI and hybridized with PCR-generated probe for *p16* exon 2 (A), *p15* exon 2 (B), and a human *BCL-2 mbr* gene probe to estimate DNA loading (C). Although cases 4-7 show *p16* deletions, all demonstrated the *p18* gene.

Southern blot analysis was performed on 99 of 117 cases. Homozygous deletions of *p16* occurred in 8 of 23 (35%) T-ALL/LBL cases. Deletions were also found in 1 of 3 (33%) B-ALL cases. Homozygous deletions were not detected in the other lymphoid tumors studied. All cases that showed homozygous deletions of *p16* also contained homozygous deletions of *p15*. None of lymphoid tumors studied demonstrated homozygous deletions of *p18* (Fig 4).

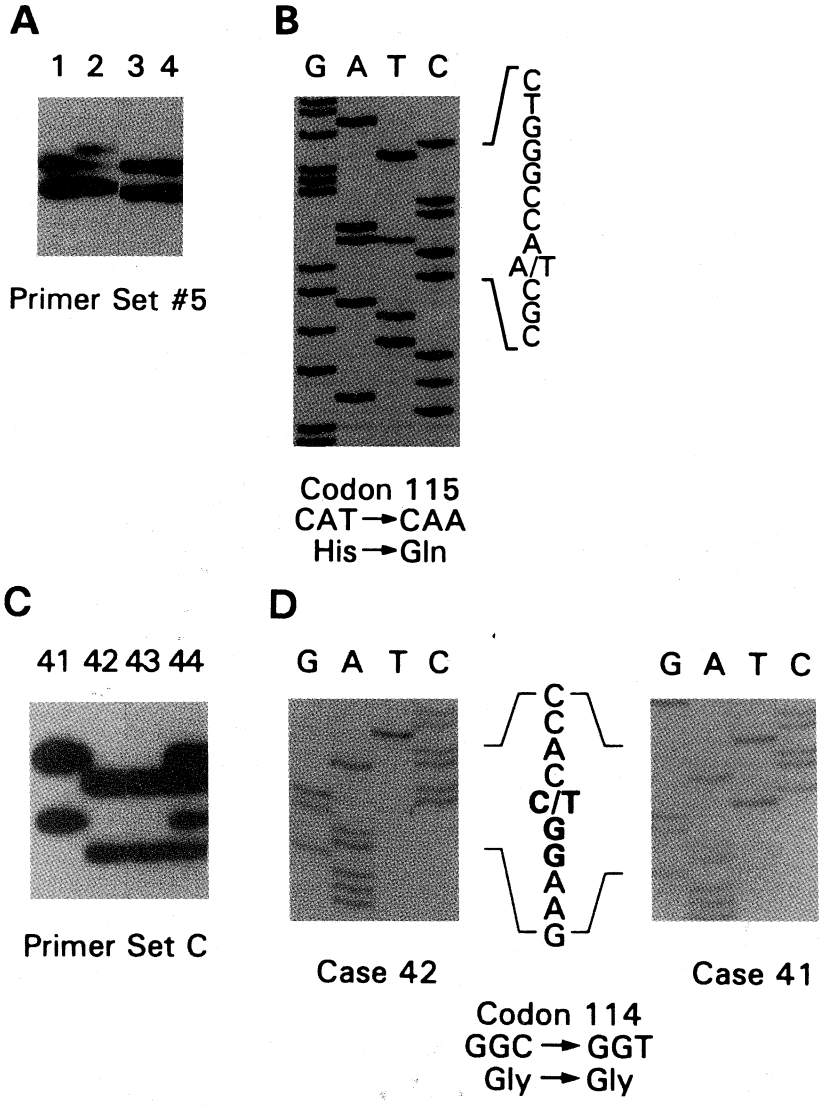

Fig 5. PCR-SSCP and sequencing analyses. A. Single abnormal conformer detected in the 3'-region of *p16* exon 2 present in one B-CLL case (lane 2). B. T→A (CAT to CAA) single nucleotide transversion in codon 115 resulting in a missense amino acid substitution changing histidine to glutamine. C. Single, recurring abnormal conformer detected in the 3' end of the *p18* gene. Homozygous pattern in lane 41, and heterozygous pattern in lane 44. D. C→T (GGC to GGT) single nucleotide transversion in codon 114, which does not result in amino acid substitution.

SSCP analysis for mutation of INK4 family CDK-1 genes. The coding region of exons 1α, 2, and 3 of *p16*, exon 2 of *p15*, and exons 1 and 2 of *p18* were studied by SSCP-PCR. Mutations were not detected in any T-ALL/LBL, nor in any of the remaining 99 lymphoid neoplasms, with two possible exceptions. B-CLL-2 displayed an abnormal conformer in the 3' region of *p16,* (Fig 5A). Sequence analysis showed a missense mutation in codon 115 (histidine to glutamine ; CAT→CAA) (Fig 5B). In addition, one T-ALL/LBL possessed a GCC→GCT (Ala→Ala) sense mutation at codon 110 of *p15*.

Two previously reported polymorphisms of *p16*, and 1 of *p18* were detected, one at codon 140 (GCG→ACG ; alanine→threonine), at a frequency of 1%, one within noncoding sequences in *p16* exon 3, at a frequency of 14%, and one in *p18* codon 114 (GGC→GGT ; Gly→Gly) at a frequency of 9.7% (Fig 5C, and D).

TABLE 1. Incidence of CDK inhibitor gene alterations in human lymphoid malignancies*

Classification		Number of Cases Studied		Deletional Analysis (Southern Blot) Number of Deleted Cases						Mutational Analysis (SSCP) Number of Positive		
Lineage	Tumor Type	*p16* and *p15*	*p18*	*p16* Deletion	%	*p15* Deletion	%	*p18* Deletion	%	*p16*	*p15*	*p18*
T	ALL/LBL	23	18	8/23	34.8	8/23	34.8	0/18	0	0	1**	0
	CLL	3	4	0/2	0	0/2	0	0/4	0	0	0	0
	PTCL	36	18	0/27	0	0/27	0	0/18	0	0	0	0
	ATL	3	0	0/2	0	0/2	0			0	0	
B	CLL	37	30	0/34	0	0/34	0	0/30	0	1***	0	0
	NHL	10	9	0/10	0	0/10	0	0/9	0	0	0	0
	ALL	5	2	1/3	33.3	1/3	33.3	0/2	0	0	0	0

*Data from Hematopathology Section, Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland, USA
**Sense mutation in codon 110 of *p15* gene (GCC→GCT, Ala→Ala)
*** Missense mutation in codon 115 of *p16* gene (CAT→CAA, His→Gln)

ALL, acute lymphoblastic leukemia; LBL, lymphoblastic lymphoma; CLL, chronic lymphocytic leukemia; PTCL, peripheral T-cell lymphoma; ATL, adult T-cell leukemia/lymphoma; NHL, non-Hodgkin's lymphoma.
All cases examined for *p18* gene were screened for *p16* and *p15* genes.

Discussion on the results from National Cancer Institute studies (Table 1). Homozygous deletions of *p16* and *p15* were found in 35% of T-ALL/LBL and in one of three B-ALL, but not in other lymphoid neoplasms. Deletions of *p18* were not detected in any case. Mutations of *p15* and *p16* were rare, and those identified do not seem to be significant, suggesting that inactivation of these genes occurs mainly through their loss. The data are consistent with a role for *p16* and/or *p15*, but not *p18*, in the pathogenesis of acute lymphoblastic leukemia/lymphoma, particularly of T-cell phenotype.

The high incidence of homozygous deletions, and the inability to detect point mutations in either *p16* and *p15* genes leads one to consider the

possibility that more than one gene in the 9p21 locus must be inactivated to provide the leukemogenic growth advantage. A requirement for inactivation of more than one closely linked gene may favor a single multilocus deletional event over the simultaneous occurrence of inactivating point mutations occurring in several genes. This would explain the low frequency of point mutations of *p15* and *p16* in the leukemias, as well as in certain other cancers.

Conclusions from the results of National Cancer Institute studies were as follows: (1) frequent (35%) homozygous deletions of the *p16* and *p15* genes were detected in acute lymphoblastic leukemia/lymphoma, particularly of T-cell phenotype suggesting that loss of function of these genes plays an important role in the pathogenesis of ALL; (2) mutations within *p15* and *p16*, are rare in ALL, suggesting that this mechanism of gene inactivation is not prominent in ALL; (3) in other types of lymphoid tumors neither deletions nor mutations of *p15* and *p16* were identified, suggesting that these CDK-Is are not commonly inactivated by these mechanisms in the other malignancies studied; and (4) neither deletion nor mutation of *p18* was detected in any lymphoid malignancy studied, indicating that *p18* loss (through these two mechanisms) is not associated with lymphomagenesis.

ALTERATION OF INK4 FAMILY CDK-I GENES IN HEMATOLOGICAL MALIGNANCIES SUMMARIZED FROM RECENT LITERATURE

Deletional analyses of CDK-I genes

Lymphoid neoplasms, particularly lymphoblastic leukemias and lymphomas, have been reported to exhibit abnormalities such as deletions and rearrangements at chromosome 9p21 by classical karyotypical analyses.[63-69] Significantly, the initial two reports identifying *p16* and *p15* as candidate tumor suppressor genes contained a total of eighteen leukemia cell lines and reported more than half of them to have homozygous loss of both *p16* and *p15*.[48,50] These findings lead others to examine the possibility that alterations of these genes could occur in primary hematological malignancies as well. Over the past few years, approximately thirty articles have been published, including our own, concerning the possible presence of CDK-I alterations in a variety of hematological malignancies.[61,62,70-100]

Table 2 (i to iv) is a summary of the published deletional analyses of *p16*, *p15* and *p18* in various hematological malignancies according to disease type. Results of deletional analyses from studies of acute lymphoblastic leukemia (ALL) are presented in Table 2-i. Homozygous deletion of *p16* and *p15* genes were frequently found in T-cell ALL/lymphoblastic lymphoma (LBL). *p16* deletions have been reported in 54.7% (162/296 cases) while *p15* deletions occurred in 50.5% (94/186 cases). The percentage of hemizygous deletions of both genes in T-ALL/LBL was less than that for homozygous deletions, occurring in 8.7% of the cases (10/115) for *p16* and 31.9% (15/47) for *p15*. B-ALL cases showed relatively less frequent homozygous deletion compared to T-ALL. *p16* was homozygously deleted in 19.5% (98/502); *p15* showed homozygous deletion in 14.3% (39 out of 272) in B-ALL. Hemizygous deletion of these genes in B-ALL occurred at a similar incidence (14.1% (30/213) for *p16* and 16.6% (25/151) for *p15*). The high incidence of *p16* and *p15* deletion in ALL is consistent with the notion that loss of function of these CDK-I

TABLE 2. Deletional analysis of INK4 family CDK- I genes in hematological malignancies

Disease :	Lineage/subtype	Total Number Studied	*p16* Type of Deletion Homo	*p16* Hemi	*p15* Type of Deletion Homo	*p15* Hemi	*p18* Homo	Ref. #
		(i) acute lymphoblastic leukemias						
ALL	T-cell phenotype	40	32	–	27	–	–	72
		12	3	–	–	–	–	73
		11	0	–	–	–	–	74
		23	8	–	8	–	–	61
		11	9	1	8	2	–	76
		11	2	–	–	–	–	77
		22	17	–	9	–	0	81
		18	–	–	–	–	0	62
		11	7	1	5	3	–	79
		54	22	–	–	–	–	82
		20	3	0	2	0	–	84
		59	44	6	35	10	–	87
		3	3	–	–	–	–	91
		7	4	2	–	–	–	92
		7	7	0	–	–	–	93
		5	1	–	–	–	–	96
			162/296	10/115	94/186	15/47	0/40	
			54.7%	8.7%	50.5%	31.9%	0%	
	B-cell phenotype	37	3	–	1	–	–	72
		39	6	–	–	–	–	73
		53	11	–	–	–	–	74
		3	1	–	1	–	–	61
		32	7	7	7	7	–	76
		47	8	–	–	–	–	77
		81	12	–	5	–	0	81
		2	–	–	–	–	0	62
		40	6	6	6	7	–	79
		79	23	11	19	11	–	84
		15	8	–	–	–	–	91
		23	1	0	–	–	–	92
		39	8	6	–	–	–	93
		14	4	–	–	–	–	96
			98/502	30/213	39/272	25/151	0/83	
			19.5%	14.1%	14.3%	16.6%	0%	
	Biphonotypic/Mixed	6	0	–	0	–	–	72
		8	0	3	0	3	–	84
	Undifferentiated/Null	4	2	–	2	–	–	72
		1	0	0	0	0	–	79
		6	4	0	4	0	–	84
	Burkitt's type	12	0	–	–	–	–	73
	Lineage not described	14	4	–	–	–	–	70
		19	5	–	–	–	–	76
		27	6	–	–	–	–	80

TABLE 2. Deletional analysis of INK4 family CDK-I genes in hematological malignancies

Disease :	Lineage/subtype	Total Number Studied	*p16* Type of Deletion Homo	*p16* Hemi	*p15* Type of Deletion Homo	*p15* Hemi	*p18* Homo	Ref. #
			(ii) myelogenous leukemias					
AML		45	0	-	-	-	-	70
		37	0	-	0	-	-	72
		11	0	-	0	-	-	73
		25	1	-	-	-	-	75
		41	0	-	-	-	-	77
		44	3	1	2	1	-	80
		134	2	0	1	0	-	84
		4	0	-	-	-	-	91
		23	0	-	-	-	-	96
			6/364	1/178	3/226	1/178	-	
			1.6%	0.6%	1.3%	0.6%		
MDS		28	0	-	-	-	-	73
		22	0	-	-	-	-	77
		13	0	0	0	0	-	80
			0/63	*	*	*	*	
			0%					
CML	Blastic Crisis	10	5	-	-	-	-	75
	: Lymphoid	12	2	0	2	0	-	84
		8	3	-	-	-	-	90
			10/30	*	*	*	*	
			33.3%					
	Blastic Crisis	24	0	-	-	-	-	75
	: Non-lymphoid	18	0	0	0	0	-	84
		9	0	-	-	-	-	90
			0/51	*	*	*	*	
			0%					
	Blastic Crisis	13	0	-	-	-	-	70
	: Lineage not described	10	0	-	-	-	-	77
CML	Accerelated Phase	3	0	0	0	0	-	84
CML	Chronic Phase	33	0	-	-	-	-	77
		28	0	0	0	0	-	84
		22	0	-	-	-	-	90
			0/83	*	*	*	*	
			0%					
	Phase not described	7	0	0	0	0	-	80
MPS		15	0	-	-	-	-	77

plays an important role in development of leukemo-lymphomagenesis in ALL, particularly in those of T-cell lineage origin.

On the contrary, most of the published studies concerning deletion of *p16* and *p15* in myeloid leukemias, including acute myelogenous leukemia (AML), myelodysplastic syndrome (MDS), chronic myelogenous leukemia (CML), and myeloproliferative disorders (MPS), have not shown any significant occurrence

TABLE 2. Deletional analysis of INK4 family CDK- I genes in hematological malignancies

Disease:	Lineage/subtype	Total Number Studied	*p16* Type of Deletion Homo	*p16* Hemi	*p15* Type of Deletion Homo	*p15* Hemi	*p18* Homo	Ref. #
(iii) chronic types of lymphocytic malignancies - leukemias and others								
ATL	Acute Phase	23	4	4	4	3	-	78
	Chronic Phase	14	1	1	0	0	-	78
	Phase not described	10	1	-	1	-	-	72
		2	0	-	0	-	-	61
		14	5	0	5	0	-	84
			11/63	5/51	10/63	3/51	-	
			17.5%	9.8%	15.9%	5.9%		
CLL	B-cell phenotype	6	1	-	1	-	-	72
		34	0	-	-	-	-	74
		34	0	-	0	-	-	61
		30	-	-	-	-	0	62
		24	0	-	-	-	-	96
	T-cell phenotype	3	0	-	0	-	-	72
		2	0	-	0	-	-	61
		4	-	-	-	-	0	62
	Lineage not described	20	0	-	-	-	-	73
		81	1	7	1	5	-	80
		14	0	-	-	-	-	77
		15	2	0	2	0	-	84
			4/233	7/96	4/141	5/96		
			1.7%	7.3%	2.8%	5.2%		
PLL	B-cell phenotype	7	0	-	-	-	-	74
	T-cell phenotype	1	0	-	0	-	-	72
		46	0	-	-	-	-	74
CLL+PLL	B-cell phenotype	50	0	-	-	-	-	92
	T-cell phenotype	20	0	-	-	-	-	92
HCL		7	0	-	0	-	-	72
		6	0	-	-	-	-	74
		7	0	0	0	0	-	80
LGL		12	0	-	0	-	-	72
		6	0	-	-	-	-	74

of homozygous or hemizygous deletions except in the lymphoid blastic crisis of CML (33.3%: 10/30 cases) as shown in Table 2-ii.

Among the non-myeloid, non ALL lymphoid leukemias, including adult T-cell lymphoma/leukemia (ATL), chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), hairy cell leukemia (HCL), and large granular cell leukemia (LGL), deletions of *p16* and *p15* genes are rare, with the exception of ATL as shown in Table 2-iii. The incidence of homozygous deletions of these genes in ATL is 17.5% for *p16* and 15.4% for *p15*. Deletion of these genes appears to be associated with the acute phase of ATL. This phenomenon seems to be similar to lymphoid blastic crisis of CML cases.

TABLE 2. Deletional analysis of INK4 family CDK- I genes in hematological malignancies

Disease : Lineage/subtype	Total Number Studied	*p16* Type of Deletion Homo	*p16* Hemi	*p15* Type of Deletion Homo	*p15* Hemi	*p18* Homo	Ref. #
(iv) chronic types of lymhpcitic malignancies-lymphomas and others							
NHL B	43	0	–	–	–	–	77
	8	0	–	0	–	–	61
	9	-	–	–	–	0	62
	31	2	–	–	–	–	74
	37	1	–	1	–	–	83
	42	3	–	–	–	–	85
	209	13	–	–	–	–	86
: LPL	6	0	–	–	–	–	96
: MCL	2	0	–	–	–	–	96
: BL	8	1	–	–	–	–	74
T	7	3	–	–	–	–	74
	27	0	–	0	–	–	61
	18	–	–	–	–	0	62
	16	1	–	0	–	–	83
Lineage not described	33	4	0	4	0	–	84
SLVL	22	0	–	–	–	–	74
		28/461	*	5/121	*	*	
		6.1%		4.1%			
HD	85	0	–	–	–	–	78
Myeloma	16	0	–	–	–	–	73

(Abbrviations used in Table 2 are following; ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; MDS, myelodysplastic syndrome; CML, chronic myelogenous leukemia; MPS, myeloproliferative disorders; ATL, adult T-cell leukemia/lymphoma; CLL, chronic lymphcytic leukemia; PLL, chronic prolymphocytic leukemia; HCL, hairly cell leukemia; LGL, large granular lymphocytic leukemia; NHL, non-Hodgkin's lymphoma; LPL, lymphoplasmacytoid lymphoma; MCL, mantle zone cell lymphoma; BL, Burkitt's lymphoma; SLVL, splenic lymphoma with villous lymphocytes; HD, Hodgkin's disease.)

For the non-Hodgkin's lymphomas, homozygous deletions of *p16* and *p15* genes have been reported in 6.1% and 4.1% of cases, respectively (Table 2-iv). Hodgkin's disease and multiple myeloma have not been reported to possess deletions.

The results of the published studies suggests that the *p16* and *p15* genes are frequently deleted in acute lymphocytic leukemias and in blastic phases of more chronic processes such as CML and ATL. In addition, most of the reported cases are of T-cell lineage and most cases which demonstrate homozygous deletion of the *p16* gene also show homozygous deletion of *p15* gene. On the contrary, deletions of *p16* and *p15* genes are rare among myeloid malignancies and mature types of malignant lymphocytes including most chronic leukemias, adult lymphomas, and myelomas. Hemizygous deletions of both genes occur in the same lymphoid tumors as are found homozygous deletions, although at a lower frequency. In addition, there have been no reported deletions of the *p18* gene in the few reports that have focused on this

CDK-I, and , to date, there are no published reports concerning alterations of *p19* in lymphoid neoplasms.

TABLE 3. Mutational analysis of CDK-I genes in hematological malignancies*

Gene	Ref. #	# of Cases Studied	# of Cases Altered	Disease	Location (Codon)	Alteration	Predicted Changes
p16	73	ALL 63	1	ALL	49	GCC→GTC	Ala→Val
		AML 11					
		MDS 28					
		CLL 20					
		MM 18					
	61	ALL 28	1	B-CLL	115	CAT→CAA	His→Gln
		CLL 40					
		ATL 3					
		NHL 46					
	77	ALL 40	0				
		NHL 43					
	79	ALL 15	(Hemi. Del. Cases)				
			1	B-ALL	5' end of exon 1	7 bp insertion	frame shift
						6 bp insertion	
	80	CLL 6	(Hemi. Del. Cases)				
			1	CLL	8 bp 5' to exon 2	27 bp deletion	Splicing of exon 2
			1	CLL	90	CAC→CGC	His→Arg
					108	GAC→GTC	Asp→Val
			1	CLL	45	ATG→ACG	Met→Thr
	81	ALL 54	0				
	82	ALL 56	1	T-ALL	58	CAC→CGC	His→Arg
			1	T-ALL	50	7 bp insertion	frame shift
			1	T-ALL	63	16 bp deletion	frame shift
						5 bp insertion	
			1	T-ALL	intron 1	5 bp deletion	
					(16 bp 5' to exon 2)	9 bp insertion	
	83	NHL 67	0				
	84	AL 74	0				
	85	NHL 52	1	B-NHL	72	CGA→CAA	Arg→Gln
			1	B-NHL	3	35 bp deletion	frame shift
			1	B-NHL	49	13 bp deletion	frame shift
			1	B-NHL	intron 1	26 bp changes	
	86	NHL 209	0				
	87	ALL 59	2	T-ALL		point mutation	
	93	ALL 6	0	(Hemi. Del. Cases.)			
	94	AL 80	0				

A few articles have focused on the relationship between clinical parameters and deletions of CDK-I genes.[73,77,81] According to these reports, homozygous deletion of *p16* gene is associated with high leukemic cell mass, high white blood cell counts (WBC), and leukemias presenting with mediastinal mass. Homozygous deletion of *p15* has also been associated with a high WBC. On

the other hand, patient survival was not affected by the presence or absence of *p16*. These are early studies and additional studies will be necessary to completely evaluate the influence of these CDK-I inhibitor alterations on the clinical features and prognosis of patients with ALL and other diseases.

TABLE 3. Mutational analysis of CDK-I genes in hematological malignancies*

Gene	Ref. #	# of Cases Studied	# of Cases Altered	Disease	Location (Codon)	Alteration	Predicted Changes
				(*continued*)			
p15	61	ALL 28	0				
		CLL 40					
		ATL 3					
		NHL 46					
	79	ALL 15	0	(Hemi. Del. Cases)			
	81	ALL 54	0				
	83	NHL 67	0				
	86	NHL 209	0				
	94	AL 80	0				
p18	62	ALL 20	0				
		CLL 34					
		NHL 27	0				
	81	ALL 54	0				

*excluding sense mutations and known polymorphisms
(Abbreviations used in Table 3 are the same as Table 2 except the following: AL, acute leukemia (lineages were not described in the original article); Hemi. Del. Cases, cases with hemizygous deletion included)

Mutational analyses of CDK-I genes

In contrast to the frequent deletion of *p16* and *p15* found particularly in the ALL cases, somatic mutations within both genes are rare. As shown in Table 3, point mutations in the coding region of *p16* gene have been reported in only 8 cases (4 ALL, 3 CLL, and 1 NHL). Relatively small nucleotide alterations such as insertions and deletions less than 50 nucleotides were observed in 5 (1 B-ALL, 2 T-ALL, and 2 B-NHL) cases. Three additional cases showed nucleotides changes in intron 1 of *p16* gene. There are no reports of mutations leading to amino acid substitutions for *p15* and *p18*. Thus, despite the fact that deletion of *p16* is frequent in the acute lymphocytic neoplasms, mutation of *p16* is not. This data contrasts with that seen in some cancers such as pancreatic and esophageal carcinoma, but is entirely consistent with data obtained from studies of other cancers such as breast cancer and high grade gliomas which also have a high incidence of deletion and a concomitant low incidence of mutation (see below).

INVOLVEMENT OF INK4 FAMILY CDK-IS IN CANCERS OTHER THAN HEMATOLOGICAL MALIGNANCIES

TABLE 4. Involvement of the p16 gene in tumors other than hematological malignancies

Tumor Type	Deletioal Analysis			Mutational Analysis		Ref. #
	LOH 9p	p16 Homo	Hemi	*p16*	*p15*	
Gliomas						
Ependymoma	0/1			0/1		101
	1/2			0/2	0/2	103
	0/5*			0/5*		113
Astrocytoma	1/7			0/7		101
		0/6				102
	4/5			0/5	0/5	103
		0/8	0/8			106
		0/7				107
Oligodendroglioma	0/2			0/2		101
	3/4			0/4	0/4	103
Oligo-astrocytoma	2/3			0/3		103
Anaplastic astrocytoma	6/13			0/13		101
		1/6	3/6	0/3		102
	1/5			0/5	0/5	103
		3/16	2/16			106
		13/25				107
	2/6			0/6		108
Glioblastoma multiforme	6/11			0/11		101
		0/9	4/9	0/7		102
	18/25			1/25	1/25	103
		9/27	3/27	1/15	0/12	105
		19/46	13/46			106
		27/46				107
	13/24			1/24		108
		6/9				112
		26/38*		0/12*		113
Unclasified		11/32	8/32	0/32		103
		10/45				110
Neuroblastoma				0/18		109
Pituitary Tumor		3/25		0/25		111
Head & Neck						
Squamous Cell Carcinoma		0/68		11/68		114
		1/19		0/19		115
Nasopharyngeal Tumor						
		0/42				117
		2/3*		0/1*		118
		7/20		0/13		118
Malignant mesothelioma		15/21		0/6		119
		5/23		0/18		120
Melanoma						
Familial Kindreds				7/18		121
				2/38		122
				13/18		123
				2/10		124
Sporadic				0/30		125

Data on cell lines are not shown. *Data on Xenografts * *Rearangement

TABLE 4. Involvement of the p16 gene in tumors other than hematological malignancies

Tumor Type	Deletioal Analysis LOH 9p	*p16* Homo	Hemi	Mutational Analysis *p16*	*p15*	Ref. #
		(*continued*)				
Lung Cancer						
Non-Small Cell (NSCLC)				4/54		129
		18/27				130
		23/33				131
	13/25			1/12	0/25	132
		15/18		1/3		
		0/25		0/25	3/25	134
				19/64		136
				0/28		137
Metastatic NSCLC		4/22		6/22	5/22	134
Small Cell (SCLC)		1/5				130
		6/55				131
		0/15		0/15	0/15	134
Metastatic SCLC		0/9		0/9	0/9	134
Unclassified		8/17				138
Breast Carcinoma		0/5		0/37		139
	13/24			1/24		140
		13/20				138
Esophageal Cancer						
Squamous Cell Ca.	21/33			5/35	1/39	141
				14/27		142
				5/24		143
				4/25		144
Adenocarcinoma	7/17			1/21	1/21	141
				1/19		143
Gastric Cancer				0/19		144
Biliary Tract Cancer				14/25		146
Pancreatic Adenocarcinoma		10/27*		11/27*		148
Colorectal Tumor		0/25*		0/25*		113
		2/19				138
Renal Cell Carcinoma		1/55				149
Bladder Cancer		42/140		0/140		150
		11/110		0/110		151
			3/110**			151
				2/39		152
		5/19				138
		6/31				153
Testicular Cancer		2/42		0/42		154
Ovarian Cancer		1/21		0/21		154
		1/50		0/50		155
		16/115		0/99		157
Endometrial Cancer		1/15		0/15		154

Data on cell lines are not shown. *Data on Xenografts **Rearangement

TABLE 5. Categorization of solid tumors according to the frequency of *p16* gene deletions and mutations

	Deletions		
	frequent	infrequent	unknown
Mutations frequent	esophageal cancer (squamous cell) pancreatc cancer		melanoma(familial) biliary tract cancer
infrequent	high grade gliomas (anaplastic astrocytoma) (glioblastoma multiforme) nasopharyngeal tumor mlignant mesotherioma non-small cell lung cancer breast cancer* bladder cancer	low grade gliomas pituitary tumors small cell lung cancer colorectal cancer testicular cancer ovarian cancer endometrial cancer	neuroblastoma melanoma(sporadic) gastric cancer
unknown	renal cell cancer		

As summarized in Table 4, there have been over fifty articles published concerning CDK-I alterations (primarily of *p16*) in a variety of solid tumors other than hematological malignancies.[101-157] These tumors can be categorized as having frequent or infrequent *p16* gene deletions or mutations, as shown in Table 5. Similar to the hematological malignancies mentioned above, most non-hematological malignancies have not exhibited high frequency of somatic mutations with a few exceptions such as esophageal squamous cancers, pancreatic adenocarcinomas, and familial melanomas. On the contrary, several types of tumors exhibit frequent homozygous deletion of *p16* gene. In particular, homozygous deletion of *p16* appears to be associated with tumors of high histologic grade,[102,107,129,158,159] especially in gliomas and bladder cancers. The association of *p16* deletion with high grade tumors of a particular tumor type is similar to that seen in the blastic crisis of CML or in the acute phase of ATL among the hematological malignancies. In addition, non-small cell lung cancer has a higher incidence of homozygous deletion than small cell lung cancer, perhaps analogous to the lineage differences seen between lymphoid and myeloid leukemias in the hematologic neoplasms.

Because of the low incidence of somatic mutations of *p16* and *p15* in solid tumors as well as in hematological malignancies, despite the presence of frequent homozygous deletions in these malignancies, the possibility has been raised that there may be an other candidate(s) tumor suppressor gene located at chromosome 9p21 loci nearby the *p16* and *p15* genes. Recent molecular biological investigations, however, have demonstrated that transfection of wild type-*p16* gene into malignant cell lines can cause growth suppression *in vitro*, while transfection with mutated or tumor derived *p16* genes has no effect in inhibiting growth. In addition, hypermethylation of CpG islands in promoter region of *p16* has recently been reported to be another potential mechanism to inactive this gene. It will be necessary to investigate the several silencing mechanisms of these CDK-I genes in primary tumors and to test relationship among retinoblastoma (Rb) gene inactivation,[161-167] alteration of enzymatic activities of cyclin/CDK complexes, and inactivation of CDK-I genes.

INACTIVATION OF THE *P16* GENE THROUGH METHYLATION

It has been recently reported that hypermethylation within a 5' CpG island is associated with transcriptional silencing of the *p16* gene in human cancers.[168] This is interesting because numerous studies of human cancers have shown that there is an association between the methylation status of a gene and its expression status. Hypomethylation has generally been associated with gene activation while hypermethylation has been associated with gene inactivation (transcriptional silencing). For example, in addition to gene mutation, hypomethylation of the ras gene is associated with its transcriptional activation.[169–172] Hypomethylation of other oncogenes such as c-myc[173,174] and bcl-2[175] have also been reported in some types of malignancies as have abnormal methylation patterns of the calcitonin gene.[176–178] On the other hand, hypermethylation, leading to gene inactivation, has been reported for tumor suppressor genes such as Rb[179,180] and p53.[181]

The initial report which showed a relationship between hypermethylation and silencing of the *p16* gene documented that *de novo* methylation of the 5' CpG island of *p16* gene was found in approximately 20% of primary neoplasms, including non-small cell lung cancers, head and neck squamous cell carcinomas, and malignant astrocytomas.[162] Thereafter, there have been several articles which have demonstrated the occurrence of hypermethylation in CDK-I genes among various types of human primary cancers including hematological malignancies.[168,182–187]

TABLE 6. Freqeuency of *p16* gene hyper methylation in malignancies

Tumor types	Methylation *p16* exon1	Expression in methylated cases	Methylation *p15*	Ref. #
Leukemia				
ALL	0/8		4/8	186
AML	0/7		6/8	186
Glioma				
Grade II	3/14	2/3		185
III	2/12	2/2		185
IV	4/16	3/4		185
Malignant Astrocytoma	4/14		4/14	168/186
Squamus Cell Carcinoma of Head and Neck	1/4			168
Nasopharengial Cancer	6/27			187
non-Small Cell Lung Cancer	7/21		0/15	168/186
Small Cell Lung Cancer	0/5		0/6	168
Breast Cancer	5/16	0/5	0/16	183
Colon Carcinoma	1/10	0/1		182
	8/20	0/8	0/19	183/186
Colon Adenoma	1/6	0/1		183
Bladder Cancer	12/18	0/12		182

As shown in Table 6, 20-60% of solid tumors, including nasopharyngeal cancers, breast cancers, and bladder cancers, contained hypermethylated 5' CpG islands in the *p16* gene. Surprisingly, some colon cancers, which show a low incidence of *p16* gene deletion and mutation, have a high incidence of *p16* gene methylation.[183,186]

On the other hand, in a single report to date, ALLs and AMLs have not shown hypermethylation of the *p16* gene.[186] However, it is worthwhile to note that hypermethylation of the *p15* 5' CpG island was frequently detected in both leukemia types.[186]

These findings indicate that hypermethylation and the consequent transcriptional silencing of the CDK-I genes, *p16* and *p15*, may be an additional mechanism of CDK-I inactivation in certain subsets of tumor types. Additional investigations examining the methylation status of the CDK-I genes in various tumor types are necessary to clarify the role of these genes in tumorigenesis.

CONCLUSION

In this article, we have described the involvement of the INK4 family CDK-I genes in hematological malignancies, focusing on results obtained from our studies at the Hematopathology Section, Laboratory of Pathology, National Cancer Institute, Bethesda, USA. Inactivation of CDK-I genes occurs primarily through gene deletion and possibly through promoter hypermethylation, and appears to play an important role in lymphoid leukemogenesis, particularly in acute lymphocytic leukemias of T-cell phenotype, T-lymphoid blast crises of CML, and in ATL. Further cellular and molecular investigations will be required to understand the biological role of the CDK-I genes in leukemic cells. Moreover, additional clinical investigations will also be required to understand the clinical consequences of CDK-I gene alterations occurring in lymphoid malignancies and other solid tumors.

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