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Frequent $p16^{INK4}$ (*MTS1*) Gene Inactivation in Testicular Germ Cell Tumors

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The molecular mechanisms responsible for the development of testicular germ cell tumors (GCTs) have not as yet been elucidated. The aim of the present study was to determine whether genetic alterations of $p16^{INK4}$ (*MTS1*) and/or cyclin-dependent kinase 4 (*CDK4*) occur in the genesis of these tumors. We have analyzed these two genes in 29 testicular GCTs, seminomas, and nonseminomas. None of the tumors showed either $p16^{INK4}$ or *CDK4* mutations. Only 1 of the 29 GCTs displayed loss of heterozygosity of the $p16^{INK4}$ gene. No homozygous deletions of $p16^{INK4}$ were detected. Evidence of hypermethylation of $p16^{INK4}$ exon 1, however, was demonstrated in 13 of the 26 (50%) GCTs analyzed. Tumor samples having exon 1 of $p16^{INK4}$ methylated expressed significantly lower levels of $p16^{INK4}$ mRNA, as analyzed by reverse transcriptase polymerase chain reaction. These results suggest that $p16^{INK4}$ inactivation plays a role in the genesis of GCTs. *(Am J Pathol 1997, 151:859–865)*

$p16^{INK4}$ (*MTS1*), a tumor suppressor gene,[1] codes for an inhibitor of cyclin-dependent kinase 4 (CDK4).[2] The gene is mutated or homozygously deleted in tumors of diverse origin.[3–13] Silencing of the $p16^{INK4}$ gene promoter by *de novo* methylation has been reported for a variety of tumors.[14–16] An alternative promoter of the $p16^{INK4}$ gene has been described, giving rise to a novel first exon (E1β) spliced to the previously described second exon of $p16^{INK4}$.[17–19] The novel protein is referred to as $p19^{ARF}$ in mouse and ORF2 in man.[17–18] Overexpression of $p19^{ARF}$ results in cell cycle arrest in mammalian fibroblasts.[18]

The $p16^{INK4}$ gene product acts as a negative regulator of cellular proliferation by interacting with CDK4 and inhibiting its kinase activity.[20] In the absence of functional p16, CDK4 binds to cyclin D, which stimulates passage through the G1 phase of the cell cycle. Recently, it has been demonstrated that a CDK4 protein with a codon 24 mutation is unable to interact with the p16 protein.[21] This represents an alternative mechanism of achieving deregulation of the cell cycle.

Testicular germ cell tumors (GCTs) are the most frequent malignant tumors in young men. The genetic alterations underlying the development of these neoplasms, especially the molecular mechanisms disrupting the cell cycle regulation during testicular tumorigenesis, have not been elucidated thus far. Recently, we have shown that GCTs overexpress wild-type *p53*, indicating resistance of tumor cells to the growth-inhibitory effects of wild-type *p53*.[22] The absence of detectable $p21^{WAF1}$ expression in GCTs suggests that *p53* is unable to activate *p21* transcription.[22] A previous study failed to find $p16^{INK4}$ gene mutations in GCTs.[23]

To determine whether $p16^{INK4}$ and *CDK4* alterations play a role during testicular tumorigenesis, we analyzed the three exons of $p16^{INK4}$ and the first exon of *ORF2* as well as codons 5 to 44 of *CDK4* by polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) and DNA sequencing in 29 seminomatous and nonseminomatous GCTs. In addition, we analyzed the methylation status and allele loss frequency of the $p16^{INK4}$ gene.

Materials and Methods

Patients

Twenty-nine patients with seminomatous and nonseminomatous GCTs of the testis were investigated. The age of the patients ranged from 2 to 62 years (average, 31.7 years). All cases had been previously investigated for *p53* alterations and $p21^{WAF1}$ and *mdm2* expression.[22]

Histopathological Analysis

Tumors were classified according to the World Health Organization International Histological Classification of tumors. Histopathological typing was performed on H&E-stained formalin-fixed sections used for diagnostic purposes. There were 14 seminomas, 6 embryonal carcinomas, 3 yolk-sac tumors, 1 teratoma, 2 embryonal

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carcinomas combined with teratoma, 2 yolk-sac tumors combined with teratoma, and 1 seminoma combined with yolk-sac tumor. The percentage of tumor cells, assessed semiquantitatively on hematoxylin and eosin (H&E)-stained frozen sections adjacent to the tissue samples used for molecular analysis, ranged from 30 to 96%. In most cases, non-neoplastic tissue was also analyzed.

Polymerase Chain Reaction (PCR)

Genomic DNA was extracted from frozen tissue using standard methods.[22] A 204-bp fragment containing exon 1 of the *p16*^{INK4} gene was amplified using the following primers: 1x, GGGAGCAGCATGGAGCCG, and 1y, AGTCGCCCGCCATCCCCT. For exon 2, three overlapping fragments, 2A (171 bp), 2B (170 bp), and 2C (169 bp), were amplified separately using the following primers: 2Ax, CTGGCTCTGACCATTCTGT, and 2Ay, AGCACCACCAGCGTGTCC; 2Bx, GACCCCGCCACTCTCACC, and 2By, AGGTACCGTGCGACATCGC; 2Cx, GATGCCTGGGGCCGTCT, and 2Cy, CAGGGTACAAATTCTCAGAT. For exon 3, the following primers were used: 3x, GTAGGGACGGCAAGAGA, and 3y, ACCTTCGGTGACTGATG, yielding a fragment of 159 bp.

A 316-bp fragment containing exon 1 of *ORF2* (Exon1β) was amplified using the following primers: 1βA, GCCTGCGGGGCGGAGAT, and 1βB, GCGGCTGCTGCCCTAGA.

A 105-bp fragment containing the codon 24 of the *CDK4* gene was amplified using the following primers: CDK4-A, AGCCAGTGGCTGAAATTGGT, and CDK4-B, CATTGGGGACTCTCACACT.

The thermal cycle profile was 30 seconds at 94°C, 45 seconds at 55°C (*p16*^{INK4} exons 2C and 3, CDK4) or 58°C (*p16*^{INK4} exons 1, 2A and 2B, ORF2 exon 1), and 45 seconds at 73°C. This cycle was repeated 30 times.

Single-Strand Conformation Polymorphism (SSCP)

The PCR products were directly subjected to nonradioactive SSCP analysis as previously described.[24]

DNA Sequencing

The PCR products of interest were cloned in the pGEM-T vector (Promega, Madison, WI) according to the manufacturer's specifications. The transformed clones were screened by PCR-SSCP, and representative clones were sequenced on a Pharmacia LKB ALF automatic sequencer (Pharmacia P-L Biochemicals, Milwaukee, WI).

Methylation Analysis of *p16*^{INK4} First Exon

The methylation status of the first exon of *p16*^{INK4} was performed essentially as described.[15] All DNA samples were individually digested with *Msp*I, *Ksp*I, and *Hpa*II, extracted with chloroform/phenol, and precipitated with ethanol before PCR. Undigested DNA was included as a positive control. The primers used for PCR amplification were 1x (GGGAGCAGCATGGAGCCG) and 1 mety (CTGGATCGGCCTCCGACCGTA). The thermal cycle profile was 30 seconds at 94°C, 45 seconds at 57°C, and 45 seconds at 73°C. This cycle was repeated 30 times. PCR products were separated on 2% agarose gels. There are two *Msp*I/*Hpa*II sites and one *Ksp*I site within the 160-bp region amplified.

Loss of Heterozygosity (LOH) Analysis

p16^{INK4} gene allele loss was assessed by two different technical approaches: 1) SSCP analysis of a two-allele *Msp*I polymorphism situated 29 bp downstream of the coding region in exon 3, as described previously,[25] and 2) analysis of six microsatellite markers, D9S171, D9S126, D9S1748, D9S1749, D9S156, and IFNa.[14,26]

RT-PCR Analysis of *p16*^{INK4} mRNA Expression

Normal and tumor tissues from 11 patients (patients 19, 21, 22, 24, 25, 27, 28, 29, 30, 31, and 33) were available for mRNA analysis. Total RNA was extracted from tissue sections using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Five hundred nanograms of total RNA were reverse transcribed using M-MLV reverse transcriptase (Promega) and an oligo dT primer (2.5 μmol/L) in 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 10 mmol/L dithiothreitol, 50 mmol/L Tris/HCl, pH 8.3, containing 1 mmol/L each dNTP and 0.5 U/μl RNasin (Promega). Fragments at the 3′ ends of the *p16*^{INK4} and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) genes cDNA were amplified simultaneously by PCR. For *p16*^{INK4}, the following primers yielding a 207-bp fragment from exon 2 to exon 3 were used: 2Cx, GATGCCTGGGGCCGTCT, and 3y, ACCTTCGGTGACTGATG. For *GAPDH*, the following primer set yielding a 238-bp fragment from exon 7 to 8 was used: GAPDH-x, TTGTCAAGCTCATTTCCTG, and GAPDH-y, AGGCCCCTCCCCTCTTC. The thermal cycle (30 seconds at 94°C, 45 seconds at 55°C, and 45 seconds at 73°C) was repeated 25 times. The PCR products were analyzed on a 3% agarose gel.

Results

p16^{INK4} Gene Sequence Alterations in GCTs

No tumor-specific migrational shift could be observed in any of the 29 GCTs by PCR-SSCP analysis of the three exons of *p16*^{INK4}. Thus, no evidence of *p16*^{INK4} gene mutation was observed.

A nucleotide substitution (GCG to ACG, Ala to Thr) at codon 140 of the *p16*^{INK4} gene was found in two different seminomas (cases 7 and 27) as well as in the corresponding normal tissues. The SSCP pattern was characterized by the presence of three aberrant bands, in addition to the single band corresponding to the normal allele. This substitution alters a *Ksp*I restriction site. Only half of the PCR product of the tumors could be digested,

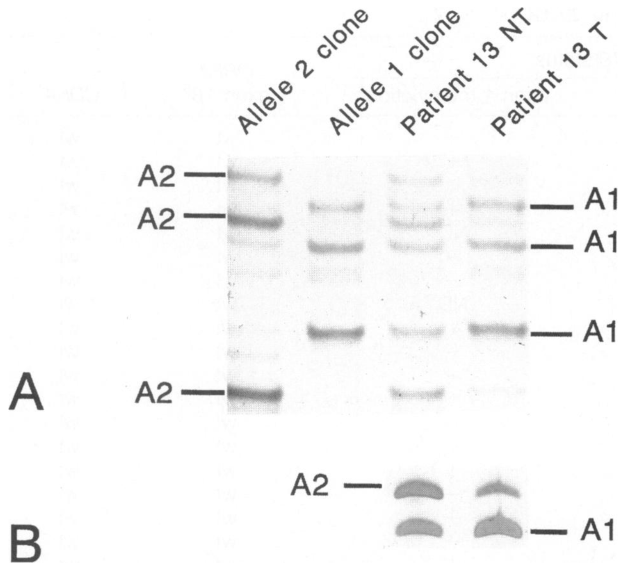

Figure 1. *MspI* polymorphism 29 bp downstream of the $p16^{INK4}$ third exon. Patient 13, heterozygous for this polymorphism, is presented. **A:** PCR-SSCP of patient 13. Tumor of patient 13 displays loss of the A2 allele. Analysis of the cloned alleles is included for comparison. **B:** The PCR product corresponding to the SSCP analysis was digested with *MspI* and separated on 12% polyacrylamide gel (silver stained). Allele loss in the tumor of patient 13 is demonstrated by the lower intensity A2 allele band. A1, allele 1 bands; A2, allele 2 bands; T, tumor; NT, non-tumor tissue.

indicating a hemizygous alteration. This sequence alteration has been described previously in different tumor types[7,9,23] and is considered a polymorphism.[9]

$p16^{INK4}$ *Gene Allele Loss in GCTs*

Seven patients (cases 7, 12, 13, 19, 23, 26, and 27) were heterozygous for a C to G germline polymorphism affecting a *MspI* restriction site identified 29 bp downstream of the coding region of $p16^{INK4}$ exon 3.[25] By SSCP analysis, this two-allele polymorphism was characterized by three additional bands (Figure 1). The PCR products were also digested with *MspI* to quantify the C (A1) and G (A2) alleles (Figure 1). In non-tumor tissues, the band intensity of the two alleles was equivalent, indicating heterozygosity. In one tumor (case 13), by contrast, the G (A2) bands were clearly weaker than the C (A1) bands, indicating G (A2) allele deletion (Figure 1). Patient 13 was informative for microsatellite marker D9S156 (see below) yet no LOH was observed.

Analysis of $p16^{INK4}$ allele loss with microsatellite markers D9S171, D9S126, D9S1748, D9S1749, D9S156, and IFNa[14,26] allowed informative analysis of all 29 patients. Figure 2 shows microsatellite results for patients 6 and 7. No additional cases with allele loss were detected (Table 2). As no LOH was observed with six microsatellite markers located at varying distances (centromerically and telomerically) from the $p16^{INK4}$ gene, homozygous $p16^{INK4}$ gene deletion could not be assessed.[26] A similar set of microsatellites allowed Cairns et al to detect small homozygous deletion of $p16^{INK4}$ in diverse primary human tumor samples.[26]

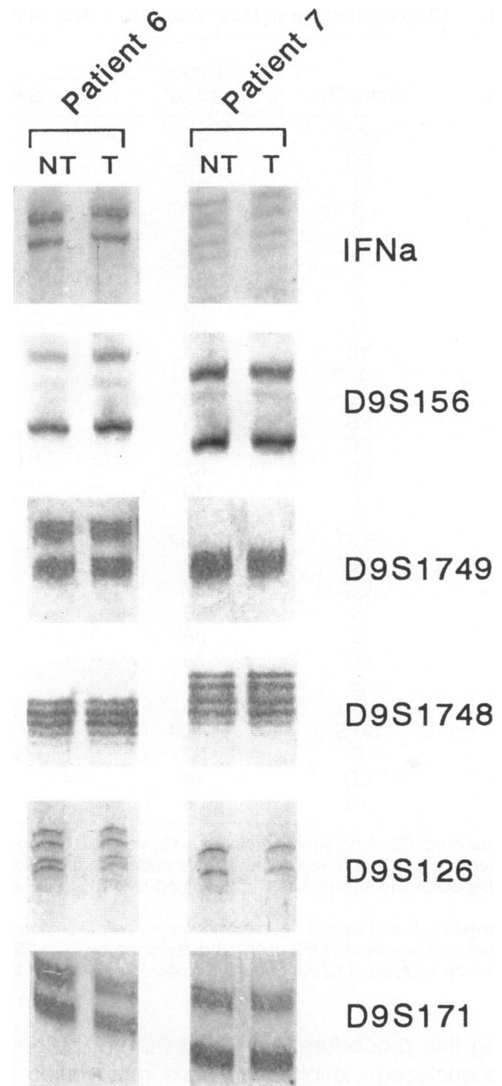

Figure 2. Analysis of tumor (T) and non-tumor (NT) tissue from patients 6 and 7 with six different microsatellite markers (D9S171, D9S126, D9S1748, D9S1749, D9S156, and IFNa). Patient 6 is informative for all markers except D9S1748 whereas patient 7 is informative for all markers except D9S1749. No LOH is evident in the tumors of these two patients.

Overall, only 1 deletion of 29 informative GCTs (3.4%) was observed.

De Novo *Methylation of Exon 1 of the* $p16^{INK4}$ *Gene in GCTs*

The $p16^{INK4}$ methylation status was analyzed in 26 of the 29 GCTs. Digestion of tumor DNAs, before PCR amplification, with methylation-sensitive restriction enzymes (*KspI* and *HpaII*) having sites within exon 1 of $p16^{INK4}$, allows the determination of methylation status of the original genomic DNA.[15] When the $p16^{INK4}$ gene exon 1 is methylated, then the restriction enzymes fail to cut and a PCR product is obtained. In this approach, undigested and non-methyl-sensitive *MspI*-digested samples serve as positive and negative controls, respectively, for each sample analyzed.

Table 1. Clinicopathological Data and p16, ORF2, and CDK4 status in 29 GCTs

Case	Histology*	Tumor cell %*	*p16* status		*ORF2* exon 1β§	*CDK4*‖
			Sequence†	exon 1 methylation‡		
1	EC/T	40	wt	−	wt	wt
2	EC	35	wt	+	wt	wt
3	S	40	wt	+	wt	wt
4	S	30	wt	+	wt	wt
5	S	53	wt	ND	wt	wt
6	S	40	wt	−	wt	wt
7	S	38	140 GCG → ACG	−	wt	wt
8	S	85	wt	ND	wt	wt
9	S	68	wt	+	wt	wt
10	YS	54	wt	−	wt	wt
11	YS/T	48	wt	−	wt	wt
12	S	80	wt	+	wt	wt
13	YS	35	wt	−	wt	wt
14	EC	76	wt	+	wt	wt
15	EC	67	wt	+	wt	wt
16	T	79	wt	−	wt	wt
17	S	57	wt	+	wt	wt
18	EC/T	58/82	wt	−	wt	wt
19	YS/T	42	wt	+	wt	wt
20	EC	57	wt	ND	wt	wt
21	S	86	wt	−	wt	wt
22	S	56	wt	−	wt	wt
23	EC	78	wt	+	wt	wt
24	S	72	wt	+	wt	wt
25	S	64	wt	+	wt	wt
26	S/YS	42/64	wt	−	wt	wt
27	S	52	140 GCG → ACG	+	wt	wt
28	EC	44	wt	−	wt	wt
29	YS	96	wt	−	wt	wt

S, seminoma; EC, embryonal carcinoma; YS, yolk-sac tumor; wt, wild-type sequence; ND, not done.
*Percentage of tumor tissue in the sample submitted to analysis.
†Sequence of *p16* exons 1 to 3 determined by PCR-SSCP screening and DNA sequencing. 140GCG to ACG, Ala to Thr polymorphism at codon 140.
‡Methylation of *p16* exon 1. +, methylation; −, no methylation.
§Sequence analysis of *ORF2* exon 1β determined by PCR-SSCP screening.
‖Sequence analysis of *CDK4* codons 5 to 44 determined by PCR-SSCP screening.

Using this procedure, we found that 13 of 26 (50%) tumors analyzed exhibited *de novo* methylation of the *p16*^{INK4} gene exon 1 (Table 1). All non-tumor tissues were unmethylated within exon 1 (Figure 3).

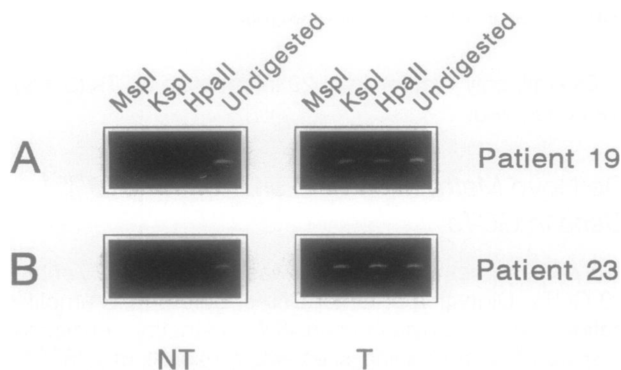

Figure 3. *De novo* methylation of the *p16*^{INK4} gene in two GCTs. Genomic DNA of non-tumor tissue (**left panel**) and tumor tissue (**right panel**) from patients 19 and 23 was digested with *MspI*, *KspI*, and *HpaII* (see Materials and Methods) before PCR amplification of *p16*^{INK4} exon 1. Undigested genomic DNA was included as a control. PCR reactions were separated on 2% agarose gels. Both non-tumor tissues are unmethylated as no amplification by PCR is seen after digestion with *KspI* and *HpaII*. In contrast, the tumor DNA from patients 19 and 23 is methylated as digestion with *KspI* and *HpaII* does not affect amplification. T, tumor; NT, non-tumor tissue.

p16^{INK4} Gene Expression by RT PCR

As methylation of the *p16*^{INK4} promoter has previously been shown to be associated with loss or lowered expression of *p16*^{INK4} mRNA, we wished to determine whether *p16*^{INK4} methylated tumor samples exhibited reduced mRNA levels. RT-PCR was performed with 11 cases, only 5 of which were interpretable (cases 23 to 27). *GAPDH* was used as an internal control. The results of these analyses are presented in Figure 4. The four *p16*^{INK4} methylated tumor samples shown (cases 23, 24, 25, and 27) all exhibited significantly lower levels of *p16*^{INK4} mRNA relative to adjacent normal tissue controls. The *p16*^{INK4} unmethylated tumor (case 26) did not have lower *p16*^{INK4} mRNA levels than the normal tissue control. Unfortunately, the RNA of the other normal tissue-tumor samples was not amenable to analysis (data not shown).

ORF2 Exon 1β Sequence and *CDK4* Codon 24 Status in GCTs

No sequence alteration was detected within *ORF2* exon 1β or at codon 24 of the *CDK4* gene by PCR-SSCP analysis in any of the GCTs (Table 1).

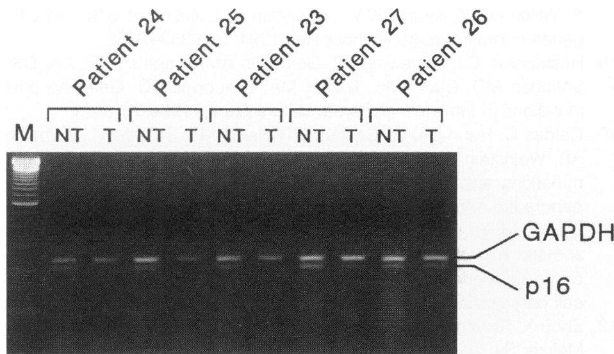

Figure 4. RT-PCR analysis of normal-tumor tissue pairs. cDNA was synthesized using oligo dT. M indicates molecular weight markers. The positions of *GAPDH* (238 bp) and *p16*^{INK4} (207 bp) PCR products are indicated. Tumors of patients 23 to 25 and 27 displayed a methylated exon 1 of *p16*^{INK4} (see Table 1). The tumor of patient 26 had an unmethylated exon 1 of *p16*^{INK4}. T, tumor; NT, non-tumor tissue.

Discussion

Little is known about the molecular changes occurring in GCTs of the testis. Mutations of the *p53* tumor suppressor gene, which are the most common genetic alterations in human malignancies, are not present in GCTs.[22] LOH at the sites of known tumor suppressor genes such as *APC/MCC*, *Rb*, and *WT-1* occur in GCTs,[27] but the functional loss of these genes has not been demonstrated. In addition, chromosomic lesions on 2q, 3p, 3q, 11p, 12p, 18q, and 22q have been observed in GCTs.[28]

The *p16*^{INK4} (*MTS1*) tumor suppressor gene is mutated or homozygously deleted in tumors of diverse origin.[3–13] Silencing of the *p16*^{INK4} gene promoter by *de novo* methylation has been observed in a variety of tumors.[14–16] Several groups have demonstrated a correlation between methylation status of exon 1 and expression of *p16*^{INK4} mRNA.[14–16] When *p16*^{INK4} exon 1 is methylated, *p16*^{INK4} mRNA is not detected, either in cell lines or in primary tumors.[14]

In agreement with a previous study,[23] we did not find any *p16*^{INK4} gene mutations in the 29 GCTs examined. However, we observed that 50% of GCTs contained a hypermethylated *p16*^{INK4} exon 1, indicating that *p16*^{INK4} gene inactivation does occur in one-half of GCTs and that the lack of *p16*^{INK4} expression might be an important mechanism leading to cell cycle deregulation in testicular neoplasms. Although the RNA of only five normal-tumor sample pairs was of sufficiently good quality to analyze by RT-PCR, there was perfect correspondence between methylation and loss of *p16*^{INK4} gene expression (see Figure 4). Our present determination of methylation analyzed two *Msp*I/*Hpa*II and one *Ksp*I sites of the first exon of *p16*^{INK4}. It is not necessarily anticipated that this methylation is functionally important, but it is a valuable diagnostic marker of *p16*^{INK4} promoter methylation.[16]

We found a hemizygous deletion of *p16*^{INK4} in only 1 of 29 cases. This LOH was not confirmed by the informative microsatellite D9S156. The latter marker is physically far away (greater than 100 kb) from the *p16*^{INK4} gene, and

Table 2. Loss of Heterozygosity in the p16 Chromosomal Region

Case	*Msp*I	D9S171	D9S126	D9S1748	D9S1749	D9S156	IFNa
1	NI	NI	NI	–	–	–	–
2	NI	–	NI	NI	NI	–	–
3	NI	–	NI	–	NI	–	–
4	NI	NI	–	NI	–	–	NI
5	NI	–	–	–	NI	–	NI
6	NI	–	–	NI	–	–	–
7	–	–	–	–	NI	–	–
8	NI	NI	NI	NI	–	–	–
9	NI	NI	NI	NI	NI	–	–
10	NI	NI	–	–	–	–	NI
11	NI	NI	NI	–	–	–	–
12	–	NI	–	NI	–	–	–
13	+	NI	NI	NI	NI	–	NI
14	NI	–	NI	–	NI	–	–
15	NI	NI	NI	–	–	–	–
16	NI	NI	NI	–	–	–	–
17	NI	–	NI	NI	–	NI	NI
18	NI	NI	–	NI	NI	–	–
19	–	–	–	–	–	–	–
20	NI	NI	–	NI	–	NI	–
21	NI	–	–	NI	–	–	–
22	NI	NI	NI	–	–	–	–
23	–	NI	NI	–	–	–	–
24	NI	–	NI	–	NI	–	–
25	NI	NI	NI	NI	NI	–	–
26	–	–	NI	–	NI	–	–
27	–	–	NI	–	NI	–	–
28	NI	–	NI	–	NI	–	–
29	NI	–	–	NI	–	–	–

Loss of heterozygosity in GCTs was determined by studying the *Msp*I polymorphism localized 29 bp downstream of *p16* exon3 or by analysis with microsatellites D9S171, D9S126, D9S1748, D9S1749, D9S156, and IFNa.
+, loss of one allele; –, no allele loss; NI, not informative.

therefore the end point of the deletion may be in the interval between the two markers. The functional significance of this allele loss remains unclear.

As one-half of the GCTs of the present series contained a normal *p16*^{INK4} gene, one can hypothesize that not only *p16*^{INK4} but also another cell cycle regulator might be altered in GCTs. Recently, it has been demonstrated that an alternative promoter of the *p16*^{INK4} gene can be used, resulting in a novel first exon (exon 1β), which is spliced to exon 2 of *p16*^{INK4}.[17–19] This alternatively spliced mRNA encodes a novel protein referred to as p19^{ARF} in mouse and ORF2 in man.[17,18] Overexpression of *p19*^{ARF} results in cell cycle arrest in mammalian fibroblasts.[18] We did not find any *ORF2* gene alterations in 29 GCTs, indicating that *ORF2* inactivation is unlikely to be involved in cell cycle deregulation during the development of GCTs.

Another potential target for cell cycle deregulation might be the *CDK4* gene. Mutations of *CDK4* codon 24 prevent p16-CDK4 complex formation in human melanoma.[21] In the GCTs of the present series, we found no evidence of *CDK4* mutation by PCR-SSCP analysis of codons 5 to 44.

An intact *Rb* tumor suppressor gene is necessary for p16 alterations to exert their effect on the cell cycle, as demonstrated previously.[29,30] Aberrations of *p16*^{INK4} and *Rb* occur in distinct subsets of human cancer cell lines.[31] Mutation and deletion of the *Rb* gene occur infrequently in testicular malignancies,[32,33] supporting the idea that *p16*^{INK4} inactivation by promoter methylation is likely to play an important role in the genesis of testicular GCTs.

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