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Homozygous Deletion of *CDKN2A* (*p16^{INK4a}*/*p14^{ARF}*) but not within 1p36 or at Other Tumor Suppressor Loci in Neuroblastoma[1]

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ABSTRACT

Loss of heterozygosity of several specific genomic regions is frequently observed in neuroblastoma tumors and cell lines, but homozygous deletion (HD) is rare, and no neuroblastoma tumor suppressor gene (TSG) has yet been identified. We performed a systematic search for HD, indicative of a disrupted TSG, in a panel of 46 neuroblastoma cell lines. An initial search focused on a well-characterized consensus region of hemizygous deletion at 1p36.3, which occurs in 35% of primary neuroblastomas. Each cell line was screened with 162 1p36 markers, for a resolution of 13 kb within the consensus 1p36.3 deletion region and 350 kb throughout the remainder of 1p36. No HDs were detected. This approach was expanded to survey 21 known TSGs, specifically targeting intragenic regions frequently inactivated in other malignancies. HD was detected only at the *CDKN2A* (*p16^{INK4a}*/*p14^{ARF}*) gene at 9p21 and was observed in 4 of 46 cell lines. The observed region of HD included all exons of both *CDKN2A* and the closely linked *CDKN2B* (*p15^{INK4b}*) gene for cell lines LA-N-6 and CHLA-174, all exons of *CDKN2A* but none of *CDKN2B* for CHLA-179, and only 104 bp within *CDKN2A* exon 2 for CHLA-101. All four deletions are predicted to inactivate the coding regions of both *p16^{INK4a}* and *p14^{ARF}*. HD was observed in corresponding primary tumor samples for CHLA-101 and CHLA-174 but was not present in constitutional samples. These results suggest that for neuroblastoma, large HDs do not occur within 1p36, most known TSGs are not homozygously deleted, and biallelic inactivation of *CDKN2A* may contribute to tumorigenicity in a subset of cases.

INTRODUCTION

The biallelic inactivation of TSGs[3] is considered crucial for the genesis and evolution of neoplastic cells. Biallelic inactivation of a specific TSG can occur by several mechanisms, including chromosomal deletion of both alleles. Although uncommon, HD is occasionally observed in primary tumors and cell lines from a variety of malignancies, and the region of biallelic deletion is usually confined to a small genomic region surrounding a target TSG. As HDs usually span relatively short genomic regions, the detection and characterization of HD in various malignancies has been instrumental in the identification of several TSGs, including *RB1*, *WT1*, and *CDKN2A* (1–3).

Neuroblastoma is a common pediatric tumor of the peripheral nervous system and is often incurable when diagnosed after 1 year of age. Neuroblastoma displays remarkable clinical heterogeneity, ranging from spontaneous regression and/or differentiation to rapidly progressive and metastatic disease, and considerable genetic heterogeneity is also apparent. Cytogenetic, molecular genetic, and functional analyses of primary tumors and cell lines have identified a number of genomic regions frequently exhibiting hemizygous deletion (4). Deletion of 1p36 correlates strongly with advanced disease and is the most well-characterized region of deletion in neuroblastoma (5–7). LOH studies have narrowed the region of consistent overlapping deletion to 1p36.3, and this region is deleted in ~35% of primary neuroblastomas (5, 8, 9). Several groups have also hypothesized the existence of one or more additional TSGs located elsewhere within 1p36 (10–12). Despite the characterization of numerous candidates, no 1p36 TSG has yet been identified, largely because of the paucity of tumors with localized 1p36 rearrangements (4).

Besides 1p36, several additional regions of the genome are frequently deleted in primary neuroblastomas, including 3p, 4p, 11q23, and 14q32 (4). However, no TSG consistently mutated or rearranged in neuroblastoma tumors has yet been identified, and analyses of several known TSGs, including *TP53*, *RET*, *CDKN2A*, and *MADH4*, have detected few if any mutations in these genes (4). Furthermore, HD of known tumor suppressor loci has been reported only rarely in neuroblastoma (13–16). The lack of evidence for genetic alterations in the genes encoding the DNA damage sensor *p53* and the cyclin-dependent kinase inhibitory protein *p16^{INK4a}* is notable, as these two genes are commonly disrupted in most malignancies (17, 18).

In the present study, we investigated whether HD occurs with significant frequency in neuroblastoma by systematically screening a large panel of cell lines with markers mapping to 1p36 and also with markers representing known TSGs. We report evidence for HD at *CDKN2A* but not within distal 1p or at other known TSGs in our cell line panel.

MATERIALS AND METHODS

Sample Collection and DNA Isolation. A panel of 46 neuroblastoma cell lines were used for HD analysis. Twenty-four cell lines have been previously described (Table 1). Cell lines CHLA-10, CHLA-101, CHLA-103, CHLA-108, CHLA-124, CHLA-132, CHLA-136, CHLA-138, CHLA-140, CHLA-143, CHLA-152, CHLA-153, CHLA-171, CHLA-174, CHLA-178, CHLA-179, CHLA-185, CHLA-52, CHLA-54, CHLA-60, CHLA-95, and CHLA-98 were established as described from patients treated with myeloablative chemoradiotherapy (19). Cell lines CHP-901 and CHP-902R were established at the Children's Hospital of Philadelphia from a bone marrow biopsy and a relapsed tumor mass, respectively, and cultured in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) with 10% fetal bovine serum, 25 μg/ml gentamicin, and 1% oxaloacetate/pyruvate/bovine insulin-media supplement (OPI; Life Technologies, Inc.). *MYCN* amplification and 1p allelic loss determinations were performed as described previously (7). Frozen tumor and corresponding normal marrow samples of patients from which cell lines CHLA-101, CHLA-174, and CHLA-179 were derived were obtained from the Children's Cancer Group Neuroblastoma Biology Resource Laboratory at Children's Hospital Los Angeles. To obtain primary tumor DNA from the patient who gave rise to the LA-N-6 cell line, cryopreserved marrow was thawed, washed to remove DMSO in Iscove's DMEM with 10% fetal bovine serum, and treated with 10 units/ml of DNase for 1 h at 37°C in a 5% CO_2 incubator. The mononuclear cells were separated using a Ficoll density cen-

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[3] The abbreviations used are: TSG, tumor suppressor gene; HD, homozygous deletion; LOH, loss of heterozygosity; SRO, smallest region of overlap; RH, radiation hybrid.

Table 1 *Neuroblastoma cell line panel*

Cell line	Reference	*MYCN* status[a]	*MYCN* reference	1p status[b]	1p reference
CHLA-10	CS[c]	Single copy	CS	LOH	CS
CHLA-42	(19)	Single copy	(19)	LOH	CS
CHLA-51	(19)	Single copy	(19)	LOH	CS
CHLA-52	CS	Amplified	CS	LOH	CS
CHLA-54	CS	Amplified	CS	LOH	CS
CHLA-60	CS	Single copy	CS	LOH	CS
CHLA-79	(19)	Single copy	(19)	LOH	CS
CHLA-90	(19)	Single copy	(19)	LOH	CS
CHLA-95	CS	Amplified	CS	LOH	CS
CHLA-98	CS	Amplified	CS	LOH	CS
CHLA-101	CS	Amplified	CS	LOH	CS
CHLA-103	CS	Amplified	CS	LOH	CS
CHLA-108	CS	Amplified	CS	LOH	CS
CHLA-123	CS	Amplified	CS	LOH	CS
CHLA-124	CS	Amplified	CS	LOH	CS
CHLA-132	CS	Single copy	CS	LOH	CS
CHLA-136	(55)	Amplified	(55)	LOH	CS
CHLA-138	CS	Amplified	CS	LOH	CS
CHLA-140	(55)	Single copy	(55)	LOH	CS
CHLA-143	CS	Amplified	CS	LOH	CS
CHLA-150	CS	Single copy	CS	LOH	CS
CHLA-152	CS	Amplified	CS	LOH	CS
CHLA-153	CS	Amplified	CS	LOH	CS
CHLA-171	(56)	Single copy	(56)	No LOH	CS
CHLA-174	CS	Single copy	CS	No LOH	CS
CHLA-178	CS	Amplified	CS	LOH	CS
CHLA-179	CS	Amplified	CS	LOH	CS
CHLA-185	CS	Amplified	CS	LOH	CS
CHP-134	(57)	Amplified	(58)	Deletion	(59)
CHP-901	CS	Amplified	CS	LOH	CS
CHP-902R	CS	Amplified	CS	LOH	CS
LA-N-5	(60)	Amplified	(61)	Deletion	(62)
LA-N-6	(63)	Single copy	(61)	LOH	CS
N206	(64)	Amplified	(65)	Deletion	(65)
NB-69	(66)	Single copy	(66)	Deletion	(66)
NBL-S	(67)	Single copy	(67)	No LOH	CS
NGP	(68)	Amplified	(58)	No deletion	(68)
NLF	(58)	Amplified	(58)	Deletion	(62)
NMB	(68)	Amplified	(58)	Deletion	(69)
SK-N-AS	(61)	Single copy	(61)	LOH	CS
SK-N-BE	(70)	Amplified	(61)	Deletion	(71)
SK-N-DZ	(72)	Amplified	(61)	No LOH	CS
SK-N-FI	(72)	Single copy	(61)	No deletion	(65)
SK-N-SH	(73)	Single copy	(58)	No deletion	(68)
SMS-KAN	(71)	Amplified	(61)	Deletion	(71)
SMS-MSN	(74)	Amplified	(61)	LOH	CS

[a] Amplified, presence of ≥3 diploid copies of the *MYCN* oncogene. Single copy, no evidence of *MYCN* amplification.

[b] Chromosomal status of 1p36. LOH, loss of heterozygosity or regional homozygosity for 1p36 polymorphisms (see "Materials and Methods"); No LOH, loss of heterozygosity or homozygosity not observed; Deletion, cytogenetic evidence for deletion of one 1p36 homologue; No deletion, no cytogenetically visible deletion of 1p36.

[c] Determined in the current study.

trifugation. Mononuclear cells were then treated with magnetic immunobeads (one bead/total cells) and tumor cells removed as described previously (20). The resulting mononuclear cells, which contained <0.1% tumor, were centrifuged to a pellet, medium-aspirated, and flash-frozen for future DNA extraction. The Jurkat T-cell line was kindly provided by S. Lessin (University of Pennsylvania). DNA was isolated from cell line pellets or frozen tissue using anion-exchange chromatography (Qiagen, Valencia, CA). DNA from Centre d'Etude du Polymorphisme Humain reference family member 1331-01 (Coriell Cell Repositories, Camden, NJ) was used as a normal control.

DNA Markers. Details of the DNA markers used to screen for HD of 1p36 and other TSGs are listed in Tables 2 and 3, respectively. Primers used for detailed mapping of HD around *CDKN2A* are included in Table 3. Primer sequences were obtained from the Genome Database[4] and from literature reports of specific TSG characterizations or were generated from a representative sequence using Primer 3.[5] If possible, TSG primers targeted gene regions commonly disrupted in other tumors. Markers for 1p36 were mapped using a distal 1p-specific mapping panel[6] as described previously. Markers mapping to 9p were ordered using map information from the HUGO chromosome 9 integrated map (Genome Database accession no. 6276683) and genomic sequence tracts from GenBank.[7]

Genotyping, HD Detection, and DNA Sequencing. A set of 46 neuroblastoma cell lines demonstrating unique genotypes at three highly polymorphic microsatellite markers (*D3S1744*, *D7S796*, and *D12S391*) were used in the HD study (Table 1). A pooled DNA template containing human RH cell lines 21-30 from the Stanford G3 panel (Research Genetics, Huntsville, AL), each of which had no human DNA fragment retention for markers within 1p36, was used as a control for detecting 1p36 HD. The 46 cell lines, a normal DNA control (1331-01), and a negative control (PCR reaction with no added template or the RH control) were amplified by PCR in 20 μl volumes containing 4 μl of 10× PCR buffer II (Perkin-Elmer, Norwalk, CT), 0.4 μM of each primer, 0.2 mM of each deoxynucleotide triphosphate, 0.2 units AmpliTaq Gold DNA polymerase (Perkin-Elmer), 1.5 mM $MgCl_2$, 0.5% Ficoll, 0.00625% bromphenol blue, and 14.4 ng template DNA. Reactions were amplified for 1 cycle at 95°C (3 min); 15 cycles at 95°C (45 s) with the annealing/extension temperature starting at 70°C and decreasing by 0.7°C each cycle (1 min); 35 cycles at 95°C (45 s), 55°C (30 s), and 72°C (1 min); and 1 cycle at 72°C (10 min). Twenty μl of each reaction solution was analyzed by electrophoresis on an enhanced sensitivity gel system (Visigel; Stratagene, La Jolla, CA), with products detected by ethidium bromide staining. PCR reactions yielding no visible signal for a specific marker were repeated, followed by radiolabeled amplification, PAGE, and autoradiographic detection as described above. DNA sequences for *CDKN2A* exon 2 were obtained on an Applied Biosystems Model 377 DNA sequencer using the ABI Taq DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer).

Southern Analysis. Ten μg of each genomic DNA sample was restriction digested for 16 h with *Pst*I (Promega, Madison, WI) and electrophoresed on a 0.6% agarose gel in 1× TBE. Gels were sequentially immersed in 0.25 M HCl for 30 min, 1.5 M NaCl/0.5 M NaOH for 30 min, and 0.5 M Tris (pH 7.4)/1.5 M NaCl for 30 min. Electrophoresed DNA was then transferred onto Hybond N^+ membranes (Amersham Pharmacia, Uppsala, Sweden), washed in 6× SSC, UV cross-linked, and hybridized to either a 1.1-kb *CDKN2A* DNA probe amplified from genomic DNA using primers CDKN2ex2-1 and CDKN2ex2-3 (Table 3) or to a probe spanning exons 3–5 of the *MLL* gene at 11q23 (kindly provided by Maureen Megonigal (Children's Hospital of Philadelphia, PA). Probe radiolabeling was performed with the Rediprime Random Labeling Kit and Redivue [α-^{32}P]dCTP (Amersham Pharmacia). Membranes were prehybridized for 3 h in 20 ml of prehybridization solution (10% dextran sulfate, 0.75 M NaCl, 0.04 $NaPO_4$ (pH 7.2), 4 mM EDTA (pH 8), 0.5% N-lauryl sarcosine, 5× Denhardt's solution, and 125 μg/ml sheared herring sperm DNA), hybridized for 16 h at 65°C after the addition of labeled probe, and washed with 2× SSC and then in decreasing concentrations of SSC-0.2% N-lauryl sarcosine at 65°C, followed by autoradiography.

RESULTS

HD Screen of 1p36. Neuroblastoma cell lines were used to simplify HD detection, because the absence of nonmalignant stromal cells allows implementation of a high-throughput true/false assay system. A set of 46 genetically distinct cell lines was selected for analysis (Table 1). Thirty-nine of the 46 cell lines (85%) had evidence of a 1p hemizygous deletion. Previously, we identified a smallest overlapping region of consistent deletion (SRO) spanning ~1 Mb within 1p36.3 by LOH analyses of primary neuroblastomas (5). This region has been further characterized by the construction and analysis of a distal 1p-specific mapping panel, which subdivides 1p35–p36 into 50 distinct genomic intervals (21). The 1p36.3 SRO is entirely contained within mapping intervals 6–11 (Table 2; Ref. 5). Seventy-six markers within the SRO, providing an average HD detection resolution of approximately 13 kb, were used to survey the 46 neuroblastoma cell lines by PCR. Included in this set of markers were primers representing each gene or expressed sequence tag cluster known to map within or near the SRO, including the proposed candidate neuroblastoma TSGs *TP73*, *TNFRSF12*, and *HKR3* (22–24). To our knowledge, HD

[4] Internet address: www.gdb.org.

[5] Internet address: www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi.

[6] Internet address: compgen.rutgers.edu/chr1/data/1p36map/index.shtml.

[7] Internet address: www.ncbi.nlm.nih.gov/.

Table 2 *Markers within 1p36 used for HD analysis*

Marker name	Marker type[a]	Map interval[b]	GDB ID[c]
Within the 1p36.3 SRO[b]			
SHGC-32565	Genomic STS	6	GDB: 6454441
D1S1215	Genomic STS	6	GDB: 9925924
TP73	Gene	6	GDB: 9925922
NBR	Gene	6	GDB: 5218325
TIGR-A008H35	EST	6	GDB: 6054525
KIAA0495-1	Gene	6	GDB: 9878921
KIAA0495-2	Gene	6	GDB: 9867124
SHGC-74093	EST	6	GDB: 9897877
D1S468	Polymorphism	6	GDB: 199719
D1S3578	EST	6	GDB: 734773
KIAA0495-3	Gene	6	GDB: 9897995
D1S1467	Genomic STS	6	GDB: 330403
D1S1341	Genomic STS	6	GDB: 330025
D1S1446	Genomic STS	6	GDB: 330340
D1S3023	EST	6	GDB: 626297
stSG4593	EST	6	GDB: 4566301
SHGC-30623	EST	6.5	GDB: 4567035
EIF3S2-1	Gene	6.5	GDB: 6054578
stSG26492	EST	6.5	GDB: 9870106
EIF3S2-2	Gene	6.5	GDB: 9868174
stSG32237	EST	6.5	GDB: 9883824
D1S2845	Polymorphism	7	GDB: 613167
D1S2132	Polymorphism	7	GDB: 685449
D1S2660	Polymorphism	7	GDB: 606855
D1S1475	Genomic STS	7	GDB: 330427
stSG10106	EST	7	GDB: 4570133
D1S3378	EST	7	GDB: 678361
WI-10480	Genomic STS	7	GDB: 1220375
D1S2893	Polymorphism	7	GDB: 614637
D1S1476	Genomic STS	7	GDB: 330430
D1S1608	Polymorphism	7	GDB: 685038
KIAA0673	Gene	7	GDB: 441576
WI-21934	EST	7	GDB: 7087737
D1S2306E	EST	7	GDB: 581241
D1S2145	Polymorphism	7	GDB: 685923
D1S3405	Genomic STS	7	GDB: 678641
D1S3676	Genomic STS	7	GDB: 3750033
stSG28971	Genomic STS	7	GDB: 6055170
D1S1371	Genomic STS	7	GDB: 330115
D1S2795	Polymorphism	7	GDB: 611883
D1S2633	Polymorphism	9	GDB: 603389
TIGR-A009F43	Genomic STS	9	GDB: 9867326
D1S47	Genomic STS	9	GDB: 549224
WI-22579	EST	10	GDB: 7087844
H96854	EST	10	GDB: 9879415
KCNAB2	Gene	10	GDB: 4565990
D1S1257	Genomic STS	10	GDB: 329773
WI-15605	EST	10	GDB: 4574060
D1S2731	Polymorphism	10	GDB: 609633
D1S3701	Genomic STS	10	GDB: 3949640
TNFRSF12-1	Gene	10	GDB: 9838558
TNFRSF12-2	Gene	10	GDB: 4565980
TIGR-A006V30	EST	10	GDB: 4588048
D20409	EST	10	GDB: 9876859
stSG28441	EST	10	GDB: 9871600
D1S3041	Polymorphism	10	GDB: 626374
D1S3702	Genomic DNA	10	GDB: 3949645
SHGC-150	EST	10	GDB: 4566749
D1S1873E	Genomic STS	10	GDB: 443043
N95458	EST	10	GDB: 9886908
stSG4588	EST	10	GDB: 4566297
stSG2139	EST	10	GDB: 4564685
U91316	EST	10	GDB: 9873033
WI-13857	EST	10	GDB: 4576676
D1S2870	Polymorphism	10	GDB: 613758
D1S253	Polymorphism	10	GDB: 188555
D1S1250	Genomic STS	10	GDB: 329752
D1S3100	Genomic STS	10	GDB: 636122
HKR3	Gene	10	GDB: 3945572
stSG22641	EST	10	GDB: 9884431
D1S1365	Genomic STS	10	GDB: 330097
D1S2642	Polymorphism	11	GDB: 603794
D1S214	Polymorphism	11	GDB: 188072
D1S1646	Polymorphism	11	GDB: 684504
D1S1398	Genomic STS	11	GDB: 330196
WI-11303	EST	11	GDB: 1220330
Outside 1p36.3 SRO[b]			
D1S3672	Genomic STS	0.5	GDB: 3750025
D1S3674	Genomic STS	0.5	GDB: 3750029
D1S1287	Genomic STS	0.5	GDB: 329863
D1S80	Polymorphism	1	GDB: 178639
TNFRSF14	Gene	1	GDB: 440310
stSG9144	EST	1	GDB: 4569901
D1S2515	EST	1	GDB: 596514
RER1	Gene	1	GDB: 4569901
D1S76	Polymorphism	1	GDB: 168840
PEX10	Gene	1	GDB: 4578612

Table 2 *Continued*

Marker name	Marker type[a]	Map interval[b]	GDB ID[c]
D1S243	Polymorphism	2	GDB: 188393
WI-16874	EST	2	GDB:4582418
WI-15347	EST	2	GDB:4579637
A006A26	STS	2	GDB:4571021
SGC34147	EST	2	GDB:4582234
WI-14412	EST	2	GDB:4581970
SCNN1D	Gene	2	GDB:5053412
SGC33169	EST	2	GDB:4580254
WI-11477	EST	3	GDB:1220275
TIGR-A007H27	EST	3	GDB:4588644
stSG9906	EST	3	GDB:4570099
WI-18288	EST	3	GDB:4582616
stSG4467	EST	3	GDB:4568853
KIAA0447	Gene	3	GDB:1220306
GNB1	Gene	4	GDB: 636086
WI-13821	EST	5	GDB:4577655
D1S97	Genomic STS	5	GDB: 636065
DVL1	Gene	5	GDB:4873202
D1S2694	Polymorphism	12	GDB: 608406
D1S1306	Genomic STS	12	GDB: 329920
D1S548	Polymorphism	13	GDB: 686691
D1S508	Polymorphism	13	GDB: 200120
DJ-1	Gene	15	GDB:1220388
TNFRSF9	Gene	15	GDB:3750021
SGC34994	EST	15	GDB:4575666
D1S1829E	EST	15	GDB: 441741
TNFRSF12-3	Gene	15	GDB:4579729
stSG4370	EST	15	GDB:4566135
D1S503	Polymorphism	15	GDB: 200109
D1S1615	Polymorphism	15	GDB: 686211
D1S3275	EST	15	GDB: 675946
SLC2A5	Gene	15	GDB: 188840
CA6	Gene	15	GDB: 636125
D1S160	Polymorphism	15	GDB: 182199
ENO1	Gene	16	GDB: 549140
TIGR-A004Y04	EST	17	GDB:4583178
FRAP1	Gene	17	GDB:1220473
D1S450	Polymorphism	18	GDB: 199528
D1S244	Polymorphism	18	GDB: 188418
PIK3CD	Genomic STS	18	GDB:1230531
D1S1768E	EST	18	GDB: 439878
SHGC-31453	EST	18	GDB:4567450
stSG1544	EST	19	GDB:4562131
D1S2028E	EST	20	GDB: 445905
TNFRSF8	Gene	21	GDB: 549221
TNFRSF1B	Gene	21	GDB: 197005
RIZ	Gene	21	GDB:4878792
SHGC-7147	Genomic STS	22	GDB:1236006
D1S1833E	EST	23	GDB: 441843
WI-13542	EST	24	GDB:4578031
D1S228	Polymorphism	24	GDB: 188240
ZNF151	Gene	25	GDB:3750046
EPHA2	Gene	26	GDB: 636074
PAX7	Gene	27	GDB: 636107
D1S3017	EST	28	GDB: 626241
HTR6	EST	29	GDB: 678346
RNU1A	Gene	30	GDB:5054970
WI-11927	EST	31	GDB:1220491
NBL1	Gene	31	GDB: 636140
ECE1	Gene	32	GDB:4581562
ALPL	Gene	33	GDB: 549237
D1S2436	Genomic STS	33	GDB: 588738
CDC42	Gene	33	GDB:1220325
RAP1GA1	Gene	33	GDB:3750043
TCEB3	Gene	34	GDB:5049874
E2F2	Gene	34	GDB: 636071
HMGCL	Gene	34	GDB: 636092
LAP18	Gene	34	GDB: 549193
ID3	Gene	35	GDB: 636098
SHGC-2516	EST	36	GDB:3802031
C1ORF4	Gene	37	GDB:9925921
CHLC.GGAA2D04	Polymorphism	38	GDB:1220268
SLC9A1	Gene	39	GDB: 549196
FGR	Gene	40	GDB: 188675
PTAFR	Gene	40	GDB: 636116
D1S247	Polymorphism	45	GDB: 188453

[a] EST, expressed sequence tag; STS, sequence-tagged site.

[b] The 1p36.3 SRO is contained within map intervals 6–11 as defined in (21). Intervals 0–15, 16–30, and 31–40 correspond approximately to cytogenetic bands 1p36.3, 1p36.2, and 1p36.1, respectively. The distal 1p36.2 translocation breakpoint in the NGP cell line maps within map interval 15. Markers are not ordered within specific intervals.

[c] GDB ID, Genome Database unique identifier.[4]

Table 3 *Tumor suppressor and 9p loci analyzed for HD*

Locus name	Cytolocation[a]	Position[b]	GDB ID[c]
Tumor suppressor loci			
VHL	3p25-p26	Exon 2	GDB: 361151
FHIT	3p14.2	Exon 6	GDB: 4579455
APC	5q21-q22	Exon 8	GDB: 439445
PTCH	9q31	Exon 11	GDB: 9925913
TSC1	9q34	Exon 15	GDB: 9925919
RET	10q11.2	Exon 7	GDB: 9925917
PTEN	10q23.3	Exon 7	GDB: 9925915
WT1	11p13	Exon 9	GDB: 371667
MEN1	11q13	Exon 3	GDB: 9925907
ATM	11q22-q23	Exon 25	GDB: 9925903
PPP2R1B	11q23	Exon 3	GDB: 9925911
BRCA2	13q12.3	Exon 9	GDB: 6013909
RB1	13q14.2	Exon 24	GDB: 344171
TSC2	16p13.3	Exon 23	GDB: 6053988
TP53	17p13.1	Exon 6	GDB: 186921
NF1	17q11.2	Exon 26	GDB: 5887052
BRCA1	17q21	Exon 8	GDB: 389047
DCC	18q21.1	3′ UTR	GDB: 190580
MADH4	18q21.1	Exon 11	GDB: 9925905
NF2	22q12.2	Exon 7	GDB: 9925909
9p markers			
D9S288	9p22-pter	0.0227	GDB: 200107
D9S286	9p22-pter	0.0471	GDB: 200054
D9S256	9p23-9p22	0.0663	GDB: 199092
D9S162	9p22	0.1122	GDB: 188003
IFNA 1&2	9p22	0.113–0.1156	GDB: 181544
IFNA 3&4	9p22	0.113–0.1156	GDB: 196683
D9S1749	9p21	0.1161	GDB: 595876
1063.7	9p21	0.1161–0.1181	GDB: 9925891
c18.b	9p21	0.1161–0.1181; 8 kb 3′ of *CDKN2A*	GDB: 9925892
CDKN2ex3	9p21	0.1181–0.1182; *CDKN2A* exon 3	GDB: 9925893
c5.1	9p21	0.1181–0.1182; *CDKN2A* intron 2	GDB: 9925895
CDKN2ex2	9p21	0.1181–0.1182; *CDKN2A* exon 2	GDB: 462451
CDKN2ex1 1+2	9p21	0.1181–0.1182; *CDKN2A* exon 1	GDB: 462445
CDKN2ex1 5+6	9p21	0.1181–0.1182; *CDKN2A* exon 1	GDB: 9925896
c5.3	9p21	0.1181–0.1182; *CDKN2A* intron 1	GDB: 9925899
R2.3	9p21	0.1181–0.1182; *CDKN2A* intron 1	GDB: 9925900
p14	9p21	0.1181–0.1182; *CDKN2A* exon 1	GDB: 9925898
R2.7	9p21	0.1183–0.1184; 4 kb 5′ of *CDKN2A*	GDB: 9925901
D9S1752	9p21	0.1183–0.1184; 11 kb 5′ of *CDKN2A*	GDB: 595993
MTS2ex2	9p21	0.1183–0.1184; *CDKN2B* exon 2	GDB: 679534
MTS2ex1	9p21	0.1183–0.1184; *CDKN2B* exon 1	GDB: 5887034
D9S171	9p21	0.1745	GDB: 188218
D9S1121	9pter-9qter	0.1828–0.1831	GDB: 685827
D9S169	9p21	0.1838	GDB: 188142
D9S319	9p21	0.1895	GDB: 228573
D9S1678	9p21	0.1906	GDB: 582665
D9S165	9p21-9q21	0.192	GDB: 188047
D9S301	9p21-9q21	0.4733	GDB: 686844

[a] Cytogenetic localizations are from the Genome Database.[4]

[b] For tumor suppressor genes, exon/intron positions of markers are listed. For 9p markers, the fractional length position or range is shown for each marker according to the HUGO chromosome 9 integrated map (GDB: 6276683). Also listed for certain 9p markers are positions relative to *CDKN2A/B*; positions were deduced from available genomic sequence.

[c] GDB ID, Genome Database unique identifier.

for 1p36 has never been reported; therefore, we created a HD control by pooling DNA from 10 RH cell lines known to contain several human DNA fragments but no fragments within 1p36. A product of the predicted size was detected with all 76 markers for every neuroblastoma cell line but not for the HD control or a negative control (no template; Fig. 1*A*).

Although all reported 1p36 deletions in neuroblastomas include allelic loss for the 1p36.3 SRO, additional 1p36 suppressor loci both distal and proximal to this region have been postulated and a number of candidate TSGs have been proposed (4). Therefore, a second set of 86 1p36 markers was used to assess whether HD might occur elsewhere within 1p36 (Table 3). Markers mapping within each of the 50 intervals defined by the distal 1p mapping panel were included to assure that each portion of 1p36 was well represented. We also designed markers for the proposed 1p36 TSGs (*TNFRSF1B*, *RIZ*, *PAX7*, *NBL1*, *TCEB3*, *E2F2*, *C1ORF4*, *LAP18*, and *ID3*; Ref. 4), as well as for twelve markers surrounding a reported 1p36.2 translocation breakpoint in the neuroblastoma cell line NGP (9, 25). The 86 markers, which provide an average 1p36 resolution of 350 kb, were used to survey the 46 neuroblastoma cell lines. A product of the predicted size was visible for each marker in every neuroblastoma cell line and in a normal control but not in a negative control.

HD Screen of Known TSGs. Several known TSGs have been characterized for mutations in neuroblastomas, but no abnormalities have been detected with significant frequency. However, most of these analyses have included only small cohorts of tumors and/or cell lines, and many identified TSGs have not been investigated. Therefore, we extended our HD search to include 21 known TSGs throughout the genome (Table 3). Intragenic primers suitable for genomic PCR were designed for each TSG. Whenever possible, primers were selected to span intragenic regions previously demonstrated to be the most frequently deleted and/or mutated in malignant cells. The 21 TSG markers were each used to survey the neuroblastoma cell line panel and the Jurkat T-cell line, which is homozygously deleted for *CDKN2A* (26). Twenty of the 21 TSG markers yielded an amplification product in every cell line. However, the *CDKN2A* primers, which span exon 2 of the gene, did not amplify a product in three neuroblastoma cell lines (CHLA-174, CHLA-179, and LA-N-6), nor in the Jurkat control, in repeated trials using both ethidium staining and radioisotopic detection (Fig. 1*B*). In addition, a *CDKN2A* product ~100 bp shorter than the predicted length was consistently generated in cell line CHLA-101 (Fig. 1*B*) and was the only product generated in this cell line.

Analysis of *CDKN2A* Deletions. The *CDKN2A* HDs detected in the four neuroblastoma cell lines were investigated further with 28 additional markers located within or flanking *CDKN2A* to map the proximal and distal HD boundaries. Included were markers spanning exons 1α, 1β, and 3 and introns 1α, 1β, and 2 of *CDKN2A*; exons 1 and 2 of *CDKN2B*; and several proximal and distal loci (Fig. 2). LA-N-6, CHLA-174, and the control Jurkat line demonstrated HD for the entire *CDKN2A* gene including exon 1β, which encodes the 5′ portion of $p14^{ARF}$, and both exons of *CDKN2B*. CHLA-179 showed HD for all exons of *CKDN2A* but not for *CDKN2B*. No additional alterations were detected for CHLA-101. A survey of the remaining 42 neuroblastoma cell lines with the four *CDKN2A* and two *CDKN2B* exon-specific markers detected products of the expected size in all cases. The LA-N-6, CHLA-174, and CHLA-179 HDs were confined to a region of 9p21 flanked by *IFNA* and *D9S171* (Fig. 2), and each deletion would be predicted to completely abolish production of both the $p14^{ARF}$ and $p16^{INK4a}$ protein products.

The *CDKN2A* HDs were then confirmed by Southern analysis (Fig. 3). Corresponding primary tumor and constitutional DNAs for LA-N-6, CHLA-174, and CHLA-179 were analyzed in parallel to determine whether HD occurred *in vivo* or only after cell-culture establishment. A *CDKN2A* exon 2 probe hybridized to the requisite 3.4-kb *Pst*I fragment in constitutional DNA samples from the three cell lines showing HD by PCR, as well as in primary tumor DNA from CHLA-179. However, in agreement with the PCR results, no hybridization was observed for the three cell lines with *CKDN2A* HD. Furthermore, primary tumor DNA for CHLA-174 also failed to hybridize to the *CDKN2A* probe, suggesting that HD occurred *in vivo* in this tumor (no LA-N-6 tumor sample was available for analysis). Hybridization with a control probe from a different chromosome to the same filter yielded a band of identical size and approximately the same intensity for each sample (not shown).

Southern analysis of cell line CHLA-101 confirmed the PCR results of a truncated *CDKN2A* exon 2 (Fig. 3). Both techniques demonstrated an ~100-bp deletion within exon 2. DNA sequencing of the

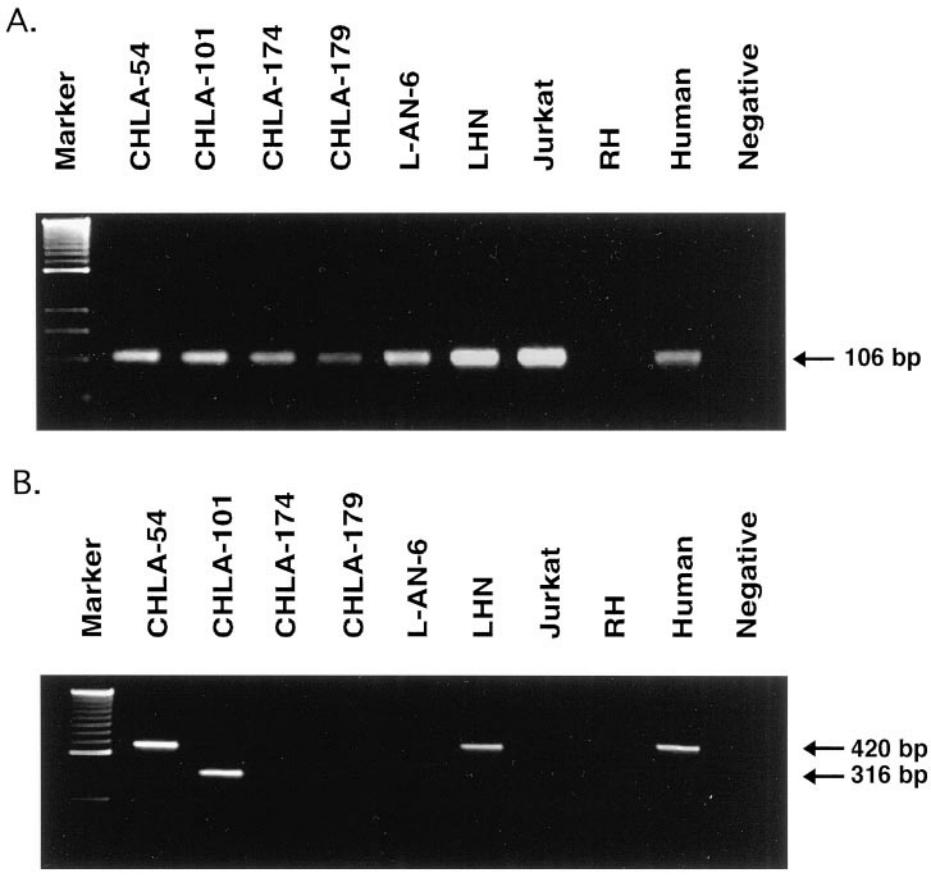

Fig. 1. Survey of HD at *D1S47* (1p36.3) and *CDKN2A* exon 2 (9p21). Cell lines were amplified with primers for *D1S47* (*A*) and *CDKN2A* exon 2 (*B*) and resolved by horizontal gel electrophoresis. *A*, at *D1S47*, a product of the predicted size was detected in every neuroblastoma cell line and in a normal human control, but not in the HD control (*RH*) or a negative control (no template). *B*, the *CDKN2A* primers did not amplify a product in three neuroblastoma cell lines (CHLA-174, CHLA-179, and LA-N-6). In addition, a *CDKN2A* product ~100 bp shorter is present only in cell line CHLA-101, which is consistent with DNA sequencing results showing a 104-bp deletion in this sample.

truncated exon 2 PCR product showed a deletion of 104 bp (bp 246–349 and residues 69–103 of $p16^{INK4a}$; and bp 390–493 and residues 83–118 of $p14^{ARF}$) entirely contained within exon 2. PCR analysis of primary tumor DNA for the patient from which CHLA-101 was derived showed only the truncated exon 2 fragment, suggesting that the primary tumor contained an exon 2 HD identical to the cell line (not shown). Sequencing of *CDKN2A* exon 2 from the primary tumor DNA confirmed these results. No corresponding constitutional DNA for CHLA-101 was available for analysis.

The 104-bp deletion of *CDKN2A* exon 2 in CHLA-101 is predicted to disrupt both $p16^{INK4a}$ and $p14^{ARF}$, which use alternate reading frames within exon 2. For $p16^{INK4a}$, the COOH-terminal 87 residues of the wild-type protein would be replaced with the 15 COOH-terminal residues of $p14^{ARF}$ because of a frameshift from the $p16^{INK4a}$ reading frame to the $p14^{ARF}$ reading frame. It is unlikely that such a fusion protein, if expressed in CHLA-101, would be functional, as the deleted region of $p16^{INK4a}$ contains the Cdk4/Cdk6 binding site where most loss-of-function point mutations localize (27). For $p14^{ARF}$, the final 50 residues would be replaced by 42 frameshifted residues.

DISCUSSION

Hemizygous deletion of distal 1p, first identified in 1977, is frequently observed in advanced stage neuroblastomas (4). However, despite the identification of a 1 Mb SRO for LOH within 1p36.3 (5), no distal 1p TSG has yet been identified. To date, >1000 primary tumors have been assessed for 1p allelic loss, but no small deletions or HD have been detected (4). Furthermore, germline, tumor, or cell line-specific 1p translocation breakpoints are rare and scattered throughout 1p (4). The lack of localized 1p rearrangements has led to alternate hypotheses for the mechanism of 1p-mediated tumor suppression. Several groups have pursued additional 1p36 TSG loci by: (*a*) mapping 1p36-specific primary tumor or cell line rearrangements identified in closely associated malignancies (28); (*b*) characterizing constitutional 1p36 rearrangements in patients subsequently developing neuroblastoma (9, 29); or (*c*) identifying correlations between 1p deletion size and the presence of *MYCN* amplification (11, 30). Moreover, although not directly supported by the present study, mechanisms other than genetically mediated biallelic inactivation of a

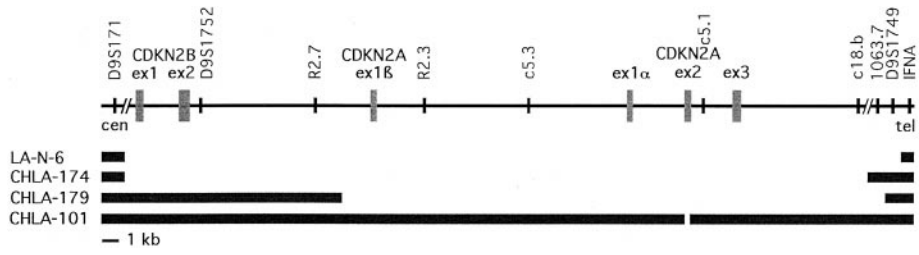

Fig. 2. Extent of HD in four neuroblastoma cell lines. *Top*, map of 9p21 region surrounding *CDKN2A*. *Shaded boxes* indicate *CDKN2B* (p15) and *CDKN2A* (p16) exons. Markers used in the HD assay are depicted by *solid vertical ticks*. The map is to scale except for the most 5′ and 3′ flanking markers and is oriented relative to the chromosome 9 centromere (*cen*) and 9p telomere (*tel*). *Below*, the extent of HD in the four neuroblastoma cell lines. *Horizontal black bars* indicate the presence of at least one copy of the region.

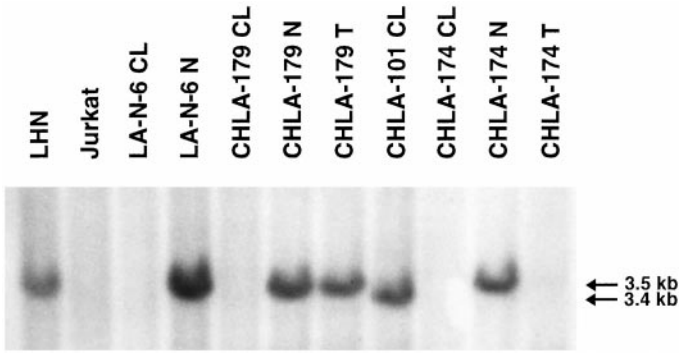

Fig. 3. Southern analysis of *CDKN2A*. Genomic DNA samples were digested with *Pst*I and hybridized with a *CDKN2A* exon 2 probe that recognizes a 3.5 kb *Pst*I fragment. HD is apparent in the cell lines Jurkat (*negative control*), LA-N-6, CHLA-179, and CHLA-174 and in a corresponding primary tumor sample for CHLA-174. CHLA-101 exhibits a band shift that is the result of a 104-bp deletion entirely within exon 2. *CL*, cell line DNA; *T*, primary tumor DNA; *N*, normal (nonmalignant tissue) DNA. No tumor sample was available for LA-N-6.

TSG may be applicable, including haploinsufficiency of one or more 1p36 loci or imprinting as an epigenetic mechanism for inactivation of the remaining TSG allele.

Our findings of no HD within 1p36 are consistent with the possibility that disruption of only a single 1p36 homologue can facilitate neuroblastoma tumorigenesis. Although the marker density we used to survey HD within the 1p36.3 SRO was high (13 kb), very small regions of HD may have gone undetected. In most cases, biallelic inactivation of a TSG occurs by intragenic deletion or bp mutation of one allele, and then by a second, larger deletion event in the remaining allele, as is common with *TP53* (31). Because we surveyed only cultured cell lines for HD at 1p36.3, it is formally possible that HD occurs more frequently in primary tumors. However, this seems unlikely, given that many of the cell lines in our study were derived from advanced neuroblastomas passaged repeatedly *in vitro*, and that most cell lines had hemizygous deletion of distal 1p.

With the exception of *CDKN2A*, HD was not detected for any of the 21 TSGs surveyed. Our inclusion of positive and negative controls for HD and the confirmation of *CDKN2A* HD by Southern analysis for all four identified HD cases suggests that our PCR-based HD assay is both sensitive and specific. It remains possible that HD of one or more TSGs went undetected with the exon primers used for TSG surveying. However, because we targeted the most commonly disrupted gene region of each TSG, it is likely that the majority of HD events would have been identified. HDs and/or inactivating mutations in neuroblastoma are known to be rare or absent in the *TP53*, *DCC*, *MADH4*, and *RET* tumor suppressor genes (4). Previously, four cases of HD have been reported for neuroblastoma: HD of several exons of the *NF1* gene at 17q11.2 in a primary tumor from a patient with neurofibromatosis type 1 (13) and in a neuroblastoma cell line (32); HD of the *CASP8* gene at 2q33 in one cell line (16); and HD of *CDKN2A* in a single cell line (see below; Ref. 14). Whereas we did not detect HD of *NF1*, the *NF1* locus has not yet been well characterized in neuroblastoma, although there appears to be no increased incidence of neuroblastoma in patients with neurofibromatosis or *vice versa* (32). The present study does not address whether HD occurs at other chromosomal loci than those included here. However, experiments using representational differential analysis did not detect HD in several neuroblastoma cell lines.[8] Furthermore, a lack of HD at a tumor suppressor locus does not preclude inactivation of the gene by other genetic or epigenetic events, such as gene mutation or methylation-mediated gene silencing.

[8] J. M. Maris and N. Lisitsyn, unpublished results.

The region of 9p21 is frequently deleted in a wide range of malignancies (33). Three loci in 9p21 have been implicated as TSGs: *CDKN2A*/$p16^{INK4a}$ and *CDKN2A*/$p14^{ARF}$, which partially share a coding exon but are encoded by two distinct reading frames; and the highly homologous *CDKN2B*/$p15^{INK4b}$ (Fig. 2; Refs. 3, 34). Substantial genetic evidence suggests that disruption of both $p16^{INK4a}$ and $p14^{ARF}$, but not $p15^{INK4b}$, is crucial for tumor development (18). Almost all tumor-specific rearrangements of 9p21 alter exon 2, which is shared by $p16^{INK4a}$ and $p14^{ARF}$, and only a few mutations affecting just one of the three implicated proteins have been reported (18, 35). Targeted deletion experiments of the three loci in mice also suggest a causative role for *CDKN2A* but not *CDKN2B*, as mice with germ-line disruptions of *CDKN2A* are cancer-prone (36). $p16^{INK4a}$ acts as an inhibitor of the cell cycle activators cdk4 and cdk6, which in turn inactivate the pRB tumor suppressor protein, whereas $p14^{ARF}$ is thought to derepress p53 by binding to and inactivating mdm2 (18). Disruptions of the *CDKN2A* locus that concomitantly eliminate functional $p16^{INK4a}$ and $p14^{ARF}$ are thus believed to inactivate both the p53 and Rb tumor suppression pathways.

Inactivation of both the Rb and p53 pathways are key events for tumorigenicity in almost all neoplasms (31, 37). Because mutations and gene rearrangements of *TP53* and *RB1* are rare in neuroblastoma, these pathways might instead be disrupted by other mechanisms. One possible alternative is a *CDKN2A* gene rearrangement that simultaneously inactivates both $p16^{INK4a}$ and $p14^{ARF}$. The four neuroblastoma HDs described in the present study all target *CDKN2A* exon 2 and disrupt both the $p16^{INK4a}$ and $p14^{ARF}$ coding regions, consistent with the type of *CDKN2A* rearrangements seen in other malignancies. The exon 2-specific 104-bp *CDKN2A* deletion observed in cell line CHLA-101 supports this hypothesis. Also, because the CHLA-101 and CHLA-179 HDs do not extend to *CDKN2B*, it is unlikely that biallelic disruption of *CDKN2B* plays a role in neuroblastoma tumorigenesis, although monoallelic deletion of this gene could conceivably have an ancillary effect. However, mutations or deletions limited to *CDKN2B* have not been detected in neuroblastomas (38).

Previous genetic analyses have reported evidence for infrequent disruption of *CDKN2A* in neuroblastoma. Several LOH studies cumulatively found 9p21 allelic loss in 29 of 131 (22%) primary neuroblastomas (14, 38–41), with LOH most frequently observed in tumors identified by mass screening of urinary catecholamine metabolites. However, HD of *CDKN2A* has not been found in primary tumors (3, 14, 38, 39, 41–43), although Dicicianni *et al.* (14) reported HD for an adriamycin-resistant subclone of the Be2C cell line that was not observed in the parent cell line. Comparative genomic hybridization studies of primary neuroblastomas have also reported only a low frequency of 9p deletions (4). Furthermore, only a single *CDKN2A* mutation, a missense mutation at residue 52 of $p16^{INK4a}$ in exon 2 has been identified out of 178 primary tumors and 28 cell lines screened (14, 38, 39, 41–43). Notably, exon 1β (encoding $p14^{ARF}$) has not been included in any of the mutation screens, but these results together suggest that a purely genetic disruption of the *CDKN2A* locus is uncommon. Our results agree with these findings in that only 4 of 46 (9%) cell lines demonstrate gross biallelic inactivation. Nevertheless, our identification of HD in two of three corresponding primary tumors suggests that *CDKN2A* inactivation is a contributing *in vivo* genetic event for a subset of neuroblastomas, rather than a strictly *in vitro* phenomenon.

It is also conceivable that $p16^{INK4a}$ and/or $p14^{ARF}$ are inactivated by alternative mechanisms. A correlation between promoter hypermethylation and decreased expression of the $p16^{INK4a}$ transcript, presumably providing an epigenetic mechanism for *CDKN2A* suppression, has been noted in other malignancies (44–46). Three studies of *CDKN2A* methylation in neuroblastoma cumulatively found exon

1α methylation in 35% of tumors and cell lines, but no correlations between methylation status and expression levels were apparent (14, 38, 41). The methylation status and mutational analysis of *CDKN2A* exon 1β in neuroblastoma has not been reported. The expression pattern of *CDKN2A* in primary tumors is varied and exhibits no obvious correlations with other parameters (38, 41, 47). Likewise, analyses of other genes in the *CDKN2A* pathway have found few abnormalities in neuroblastoma (14, 38, 42, 47–49).

Mutation of the *TP53* gene is very rare in primary neuroblastomas, and deletion or LOH of 17p is uncommon (4). There is, however, evidence that inactivation of the p53 pathway is important in some neuroblastomas. Amplification of the p53-inhibitory *MDM2* locus has been identified in several neuroblastoma cell lines and in a single tumor (50–52). Furthermore, immunohistochemistry studies suggest that p53 is sequestered in the cytoplasm in neuroblastomas, which has been postulated as a posttranslational mechanism for functional repression of p53 (53, 54). The *CDKN2A* deletions observed in the present study, which would be predicted to affect p53 function through $p14^{ARF}$, are consistent with alterations in p53 signaling playing a role in the tumorigenesis of some neuroblastomas. More detailed analysis of $p14^{ARF}$ expression and function in neuroblastoma will be needed to fully understand the contribution that this protein plays in neuroblastoma.

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