Title

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Journal

Recent Advances and Research Updates, 7(1)

ISSN

0972-4699

Author

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Publication Date

2006

IRPC-Recent Advances and Research Updates-ISSN 0972-4699

INTERNATIONAL RESEARCH PROMOTION COUNCIL

Announces

World Scientists Forum International Awards

in

Medical Genetics and Oncology

EMINENT SCIENTIST OF THE YEAR 2006

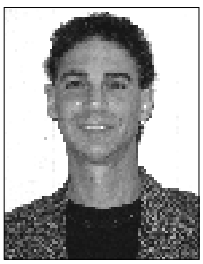
NORTH AMERICA

DR. MITCHELL B. DICCIANNI

USA

EMINENT SCIENTIST OF THE YEAR 2006

NORTH AMERICA



Dr. Mitchell B. Diccianni, Ph.D

Dr. Mitchell B. Diccianni, an expert in Pediatric Oncology is currently working as an Assistant Research Scientist at the University of California in San Diego, Department of Pediatrics Hematology/ Oncology, where he has been a faculty member since 1999.

Dr. Diccianni obtained his Bachelors of Science degree in Biochemistry in 1982 from Stony Brook University in New York. In 1989, he obtained a Ph.D. Pharmacology program at the University of Cincinnati, where he conducted thesis research in cardiovascular pharmacology under the direction of Drs. Judith Harmony and Arnold Schwartz. From 1990-1992, Dr. Diccianni was a visiting scientist at Tokyo University, where he made the tenacious switch from cardiovascular pharmacology to cancer molecular biology under the guidance of internationally acclaimed cancer researcher Dr. Masami Muramatsu. Since 1992, Dr. Diccianni has been at the University of California, San Diego in the Department of Pediatrics Hematology/Oncology. He has authored/ co-authored more than 28 peer review publications in top research journals, 35 abstracts and 5 review articles/ proceedings. His collaborations range from near and far and include scientists at UCSD and throughout the USA, and internationally with scientists from Europe and Asia.

Dr. Diccianni is also a member of the Moores UCSD Cancer Center, the international cooperative organization Childrens Oncology Group, the American Association of Cancer Research and American Society of Hematology. His research interests are primarily centered on the pediatric cancers T-cell acute lymphoblastic leukemia and neuroblastoma. His laboratory has focused on characterizing tumor suppressor genes involved in these cancers as they relate to diagnosis, prognosis and epidemiology. His laboratory has also focused on translational biology, where he has been exploiting the unique molecular profiles of cancer cells for tumor cell-selective therapeutic interventions.

Dr. Diccianni has been honored twice by the San Diego Padres baseball club for his contributions to childhood cancer research. He was selected as Honorary Scientist at the annual San Diego Easter Parade in 2005. He has been interviewed as an expert in childhood cancer by both the print and TV media, where he has promoted the merits of pediatric research and the advances UCSD scientists have made to cancer research.

Dr. Mitchell B. Diccianni has been selected by the World Scientists Forum for "Eminent Scientist of the Year 2006" International Award from USA in the field of Medical Genetics and Oncology based on his academic excellence, research contributions and expertise in the field of cancer research and pediatric blood malignancies.

UNIVERSITY OF CALIFORNIA, SAN DIEGO

The University of California San Diego is nestled along the Pacific Ocean on 1,200 acres of coastal woodland in Southern California. Since its founding in 1962, UCSD has become one of the top institutions in the nation for higher education and research. The National Research Council ranks UCSD 10th in the nation in the quality of its faculty and graduate programs, while the Institute for Scientific Information (ISI) ranks UCSD as the 9th most-cited institution in the world, based on its published research in science and the social sciences from 1995-2005. Established in 1979, the Moores UCSD Cancer Center is one of just 39 National Cancer Institute-designated Comprehensive cancer centers in the United States. As such, it ranks among the top centers in the nation conducting basic and clinical cancer research, providing advanced patient care and serving the community through outreach and education programs. UCSD is the only Comprehensive Cancer Center in San Diego and Imperial counties to have earned this honor. In 2005, the Cancer Center moved into its new building on the east side of the UCSD campus, uniting the Cancer Center's clinical enterprise (including magnetic resonance imaging, digital imaging, and radiation oncology) with basic, clinical, translational, and cancer prevention research. The Cancer Center's mission is to translate promising scientific discoveries into new and better options for the prevention, diagnosis and treatment of cancer, and for the amelioration of pain. It ranks among the top centers in the nation conducting basic and clinical cancer research, providing advanced patient care and serving the community through outreach and education programs. The Center supports research that reveals critical new insights into how cancer starts and spreads, the translation of that knowledge into promising new treatments, from prevention studies that are helping more people to lead healthier lives, and community outreach programs to bring lifesaving cancer information to underserved populations.

(The description and the accompanying photo of UCSD are summarized from the UCSD web sites http://www.ucsd.edu *and* http://cancer.ucsd.edu).

The Enigmatic Role of p16$^{CDKN2/INK4a}$ in Neuroblastoma

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ABSTRACT

Neuroblastoma is one of most common solid tumors of young children and has a wide spectrum of clinical and biological features. Most patients with localized disease (stage 1 and 2) and stage 4s survive the disease while patients with advanced disease (stage 3 and 4) have a poor prognosis. A number of molecular alterations have been associated with a poor prognosis in neuroblastoma, including amplification of N-myc proto-oncogene and deletion of a portion of the short arm of chromosome 1. Though the exact mechanism by which these alterations influence outcome remains elusive, they are known to act through cell cycle deregulation. Cyclin dependent kinase inhibitors such as p16, p18 and p27 also regulate the cell cycle. Deletions, mutations, promoter hypermethylation or translational inactivation of these genes being very common in most cancers, with p16 appearing to be almost universally inactivated in many cancer types. However, neuroblastoma is a notable exception. In contrast to inactivation, the p16 gene is paradoxically highly expressed in many advanced neuroblastoma and associated with a poor outcome. In this review, I will offer an overview of the status of the CDKI p16 in neuroblastoma, and offer some insights into the role p16 and two other CDKIs, p18 and p27, may play in this disease.

Abbreviations and Keywords: *p16 (aka CDKN2A, INK4a, MTS1); p15 (aka CDKN2B, INK4B, MTS2); p27 (aka CDKN2B, Kip1); p18 (aka CDKN2C, INK4C); CDK, cyclin dependent kinase; CDKI, cyclin dependent kinase inhibitor; HLH, helix–loop–helix; MYCN, N-Myc proto-oncogene; Neuroblastoma; Prognosis; Methylation; 9p21; Id2; ARF.*

I. NEUROBLASTOMA BIOLOGY

Neuroblastoma is a malignant tumor comprised of undifferentiated neuroectodermal cells derived from the neural crest. It is the most common extracranial solid tumor of childhood, accounting for about 10 percent of all pediatric cancers. Though the etiology of neuroblastoma is unknown, a number of genetic alterations have been associated with the disease, including deletions of 1p or 11q, unbalanced gain of 17q and amplification of N-myc (MYCN), each of which have adverse prognostic significance for neuroblastoma (Brodeur, 2002).

Neuroblastoma can broadly be divided into two categories based on biological and molecular characteristics and prognosis. Advanced stage disease (stage 3 and 4) accounts for about 60% of neuroblastoma patients and is commonly associated with metastases in bone or bone marrow and amplification of *MYCN* proto-oncogene. Survival of stage 3 patients without *MYCN* amplification can be >80% with surgery and chemotherapy. Prognosis of stage 3 patients with *MYCN* amplification and stage 4 patients is less than 50% in spite of intensive multimodality treatment including bone marrow transplantation (Castel & Canete, 2004).

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Contrasting the poor prognosis of stage 3 and 4 neuroblastoma is the favorable prognosis observed for patients with localized disease (stage 1 and 2) and stage 4s disease. Stage 1 and 2 neuroblastoma patients without *MYCN* amplification respond favorably to surgery and/or chemotherapy, with eradication of the disease obtained in greater than 90% of the patients. Stage 4s tumors are particularly unique as patients survive with little or no cytotoxic therapy due to the spontaneous regression of the disease or, occasionally, the differentiation of the tumor into benign ganglioneuroma.

II. CELL CYCLE REGULATION

Progression through the early G_1–S phase of the cell cycle depends on the interaction of the cyclin D (*D1*, *D2* and *D3*) proteins with cyclin dependent kinases (CDK) 4 and -6 (Figure 1), which in turn results in the hyperphosphorylation of the retinoblastoma (pRb) protein and the release of sequestered transcription factors such as E_2F. The CDK4/cyclin D enzyme complex is negatively regulated by CDK inhibitors (CDKIs) such as p16, p15 and p18. Passage through late G_1–S and G_2 requires the interaction of cyclin E with CDK2, which is negatively regulated by the CDKI p27. P16 and p27 also negatively regulate the cell cycle in a cooperative fashion. In the G_1 phase, p27 can bind and *stabilize* the cyclin D1/CDK4 complex, facilitating pRb phosphorylation and entry into the early G_1 phase of the cell cycle. Upon binding and inhibition of CDK4/cyclin D1 by p16, p27 is released and becomes available for binding and inhibition of CDK2/cyclin E or CDK2/cyclin A1, complementing early p16-induced G_1 inhibition with late p27-induced G_1 and G_2 phase inhibition (Bouchard et al., 1999; McConnell et al., 1999; Perez-Roger et al., 1999).

As CDKIs act as a brake on cell cycle progression, their inactivation can lead to runaway proliferation. Consistent with this hypothesis, initial investigations of *p16* status

Figure 1: Cooperative cell cycle regulation by p16 and p27

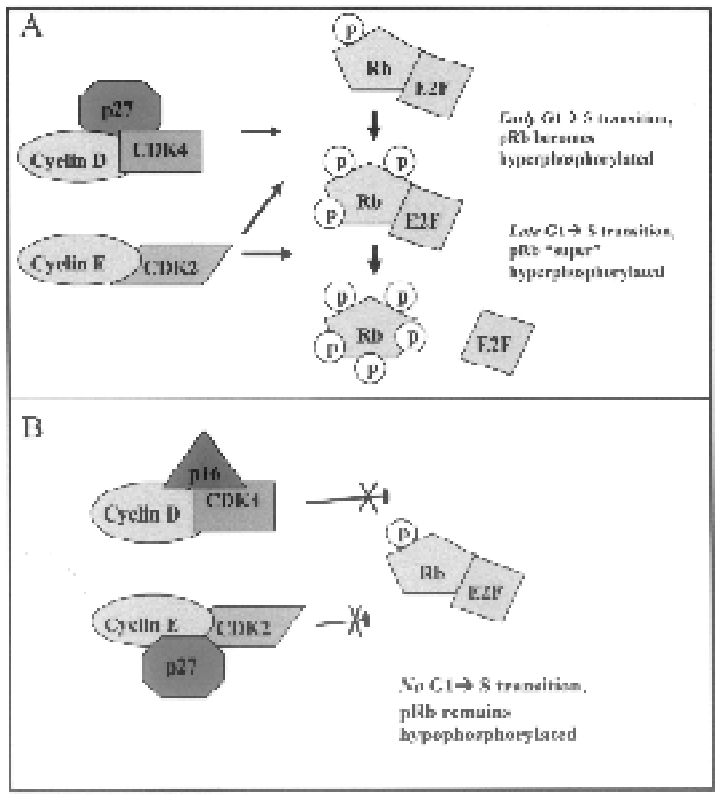

Figure 1: CDK and CDKI regulated cell cycle transition. ***A)*** During *early* G1 S transition, a p27 stabilized CDK4/Cyclin D complex results in pRb hyperphosphorylation and transition into the cell cycle. In *late* G1 S, complexes of CDK2/Cyclin E "super" hyperphosphorylated pRb completing transition into S phase. These complexes also help drive the cell through S phase into G2. ***B)*** The introduction of CDKIs such as p16 releases p27 and concomitantly inhibits the CDK4/cyclin D complex, preventing the phosphorylation of pRb and resulting in inhibition of cell cycle transition. P27 is now available for inhibition of CDK2/cyclin E or Cyclin A1 complexes, inhibiting a second mechanism of pRb phosphorylation and inhibiting cell cycle transition through G1 S and S G2.

revealed homozygous deletions in all tumor cell lines investigated with the exception of neuroblastoma and colon cancer (Kamb et al., 1994; Nobori et al., 1994). Unlike most tumor suppressor genes, mutation of *p16* is infrequent with the exception of melanoma and pancreatic carcinoma (Caldas et al., 1994; Hussussian et al., 1994). In lieu of deletion or mutation, an additional mechanism of p16 gene inactivation occurs through the aberrant methylation CpG islands located in the promoter regions, resulting in transcriptional silencing (Baylin, 2005).

The high frequency and multiple mechanisms of p16 inactivation suggest that loss of p16 is a critical early event in tumor progression.

III. P16 INACTIVATION IS INFREQUENT IN NEUROBLASTOMA

Initial studies of *p16* in neuroblastoma, which failed to find deletions or mutations, investigated only four cell lines (Kamb et al., 1994). However by 1995, only one year after its initial characterization as a tumor suppressor gene, *p16* was being widely reported to be inactivated in virtually every tumor type investigated (Sherr & Roberts, 1995). This suggested a more through investigation of *p16* status in neuroblastoma was warranted. However, subsequent analyses also failed to identify molecular alterations of the *p16* gene in neuroblastoma. An analysis of *p16* in 9 neuroblastoma cell lines and 18 primary tumors failed to identify any mutations or deletions (Beltinger et al., 1995), results that were further confirmed in a separate study of 25 primary neuroblastoma (Kawamata et al., 1996).

With reports implicating p16 in neural differentiation (Lois et al., 1995), and with the growing literature that in the absence of deletion or mutation, *p16* inactivation through promoter hypermethylation was common, we felt it was important to conduct a comprehensive molecular analysis of *p16* in neuroblastoma cell lines. In an analysis of 19 neuroblastoma cell lines, our investigations revealed for the first time a deletion of the *p16* gene (Diccianni et al., 1996). No mutations in the coding region of the *p16* gene were detected, and no promoter hypermethylation was detected (see also next section). Of five primary samples investigated in this study, and of 129 primary neuroblastoma tumors samples from various neuroblastoma stages in subsequent studies, we identified no other deletions or mutations of the *p16* gene (Gebauer et al., 2004; Omura-Minamisawa et al., 2001). Since these initial reports, studies of neuroblastoma cell lines and patient populations from around the world

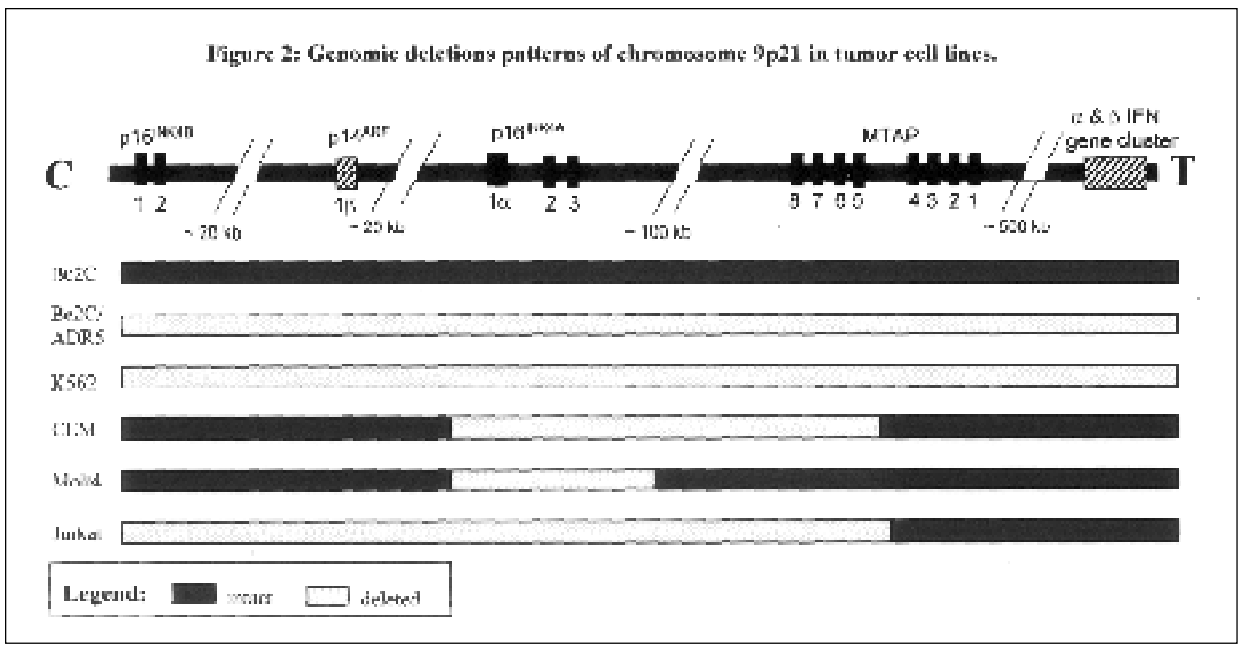

Figure 2: Genomic deletion patterns of chromosome 9p21 in tumor cell lines. The p16 gene is located on chromosome 9p21 in close proximity to at least 2 other tumor suppressor genes (*p15* and *ARF*), the metabolic gene methylthioadenosine phosphorylase (*MTAP*) and the interferon α and β (*IFN*) gene cluster. This locus in general, and *p16* in particular, is a frequent target of recombinant events that result in the deletion of one or more genes of this region. Several common patterns of deletion are outlined in this figure. *P16* is selectively deleted in the Molt4 cell line, while a deletion of >800 kb in the Be2c/ADR5 and K562 cell lines results in the loss of all genes. The CEM cell line harbors a deletion that encompasses *p16* and a portion of the *MTAP* gene, while the 9p21 deletion in the Jurkat cell line extends this deletion to include *ARF* and *p15*.

including Japan (Takita et al., 1997), Europe (Castresana et al., 1997) and South America (Bassi et al., 2004) have only confirmed the paucity of *p16* alterations in neuroblastoma.

As mentioned above, we observed homozygous deletion of *p16* in a single neuroblastoma cell line. This cell line was unique in that it was selected for adriamycin resistance, while the adriamycin-sensitive parent cell line was notably *p16* intact (Diccianni et al., 1996). A characterization of chromosome 9p21 in this cell line revealed a region of deletion extending more than 700 kb telomeric of *p16* through the interferon (*IFN*) gene cluster, and at least 100 kb centromeric of *p16* to include the *p15* gene (Figure 2). The extensive region of deletion precludes any definitive association of p16 inactivation and adriamycin resistance. The only other cell line of any tumor type with a similarly large deletion is the K562 leukemia cell line (unpublished data). We also identified a C A mutation two base pairs upstream of the p16 translation start site in a neuroblastoma cell line that highly expressed *p16* (Diccianni et al., 1999). However, the failure to find this mutation in any other cell line, *p16* expressing or not, sheds uncertainty on its role in *p16* expression. A single report of a missense mutation at codon 52 (M52K; ATG (Met) to AAG (Lys)) has been reported in a primary neuroblastomas sample (Takita et al., 1997). Though no functional data on this mutation is available, it is likely to be functionally significant due to its localization in a highly conserved region of the second ankryn repeat of p16 (Greenblatt et al., 2003). In separate analyses of *p16* in neuroblastoma cell lines, Easton et al. found two cell lines of 16 harboring homozygous deletions of p16 (Easton et al., 1998), while Thompson et al. identified 4 of 46 neuroblastoma cell lines harboring homozygous deletion of *p16* (Thompson et al., 2001). Table 1 provides for the first

Table1:
Summary of p16 gene and expression status in neuroblastoma cell lines.

Total # of cell lines	# of cell lines non-overlapping	cell line	Mutation?	Deletion?	CH3?	RT-PCR	WB	N-Myc	Pub. year	Reference
1	1	LAN-5	wt	intact	nd	nd	nd	Amp	1995	Beltinger et al. (1995)
2	2	LHN	wt	intact	nd	nd	nd	not Amp	1995	Beltinger et al. (1995)
3	3	NAB	wt	intact	nd	nd	nd	Amp	1995	Beltinger et al. (1995)
4	4	NBL-S	wt	intact	nd	nd	nd	not Amp	1995	Beltinger et al. (1995)
5	5	NGP	wt	intact	nd	nd	nd	Amp	1995	Beltinger et al. (1995)
6	6	NLF	wt	intact	nd	nd	nd	Amp	1995	Beltinger et al. (1995)
7	7	SK-N-SH	wt	intact	nd	nd	nd	not Amp	1995	Beltinger et al. (1995)
8	8	SMS-KAN	wt	intact	nd	nd	nd	Amp	1995	Beltinger et al. (1995)
9	9	SMS-KCN	wt	intact	nd	nd	nd	Amp	1995	Beltinger et al. (1995)
10	10	Be2C	wt	intact	no	++	-	Amp	1996, 1999	Diccianni et al. (1996, 1999)
11	11	Be2C/ADR5	na	deleted	na	-	-	Amp	1996, 1999	Diccianni et al. (1996, 1999)
12	12	IMR32	wt	intact	no	-/+	-	Amp	1996, 1999	Diccianni et al. (1996, 1999)
13	13	IMR6	wt	intact	no	nd	nd		1996	Diccianni et al. (1996)
14	14	NB4	mut ‡	intact	no	+++	+++	Amp	1996, 1999	Diccianni et al. (1996, 1999)
15	15	NB5	wt	intact	no	+++	+++	Amp	1996, 1999	Diccianni et al. (1996, 1999)
16	16	NB14	wt	intact	no	+++	+++	Amp	1996, 1999	Diccianni et al. (1996, 1999)
17	17	NB17	wt	intact	no	+++	+++	Amp	1996, 1999	Diccianni et al. (1996, 1999)
18	18	NB20	wt	intact	no	+++	+++	Amp	1996, 1999	Diccianni et al. (1996, 1999)
19	19	NMB7	wt	intact	no	-	-		1996, 1999	Diccianni et al. (1996, 1999)
20	20	PCL1643	wt	intact	no	-/+	-	Amp	1996, 1999	Diccianni et al. (1996, 1999)
21	21	PCL1691‡‡	wt	intact	nd	+++	+++	Amp	1996, 1999	Diccianni et al. (1996, 1999)
22	22	PCL1771	wt	intact	nd	nd	nd		1996	Diccianni et al. (1996)
23	23	PCL2021	nd	intact	nd	+	-	Amp	1999	Diccianni et al. (1999)
24	24	PCL3014	wt	intact	no	+	-	Amp	1996, 1999	Diccianni et al. (1996, 1999)
25	25	PCL3091	wt	intact	no	nd	nd		1996	Diccianni et al. (1996)

26	26	PCL4199‡‡	nd	intact	nd	+++	+++	Amp	1999	Diccianni et al. (1999)
27	27	SK-N-MC	wt	intact	no	nd	nd	not Amp	1996	Diccianni et al. (1996)
28	X	SK-N-SH	wt	intact	no	-/+	-	not Amp	1996, 1999	Diccianni et al. (1996, 1999)
29	X	SMS-KAN	wt	intact	nd	+	-	Amp	1996	Diccianni et al. (1996)
30	28	SMS-KANR	wt	intact	nd	nd	nd	Amp	1996	Diccianni et al. (1996)
31	X	SMS-KCN	nd	intact	nd	-/+	-	Amp	1999	Diccianni et al. (1999)
32	29	SMS-KCNR	wt	intact	nd	-	nd	Amp	1996	Diccianni et al. (1996)
33	30	SMS-SAN	nd	intact	nd	-	-		1999	Diccianni et al. (1999)
34	31	CHP-134	wt	intact	no	+*	+*	Amp	1997	Takita et al. (1997)
35	32	GOTO	wt	intact	no	-	-		1997	Takita et al. (1997)
36	X	IMR32	wt	intact	no	-	-	Amp	1997	Takita et al. (1997)
37	33	KP-N-NS	wt	intact	no	-	-		1997	Takita et al. (1997)
38	34	LAN-1	wt	intact	no	+*	+*		1997	Takita et al. (1997)
39	35	LAN-2	wt	intact	yes	-	-		1997	Takita et al. (1997)
40	X	LAN-5	wt	intact	no	-	-	Amp	1997	Takita et al. (1997)
41	36	NB1^	wt	intact	no	-	-		1997	Takita et al. (1997)
42	37	NB16^	wt	intact	no	-	-		1997	Takita et al. (1997)
43	38	NB19^	wt	intact	yes	-	-		1997	Takita et al. (1997)
44	39	NB39	wt	intact	no	-	-		1997	Takita et al. (1997)
45	40	NB69	wt	intact	no	-	-		1997	Takita et al. (1997)
46	41	NB9	wt	intact	yes	-	-		1997	Takita et al. (1997)
47	42	SCMCN2	wt	intact	no	+*	+*		1997	Takita et al. (1997)
48	43	SCMCN3	wt	intact	no	+*	+*		1997	Takita et al. (1997)
49	44	SCMCN4	wt	intact	yes	-	-		1997	Takita et al. (1997)
50	45	SCMCN5	wt	intact	yes	-	-		1997	Takita et al. (1997)
51	46	TGW	wt	intact	no	+*	+*		1997	Takita et al. (1997)
52	47	TNB-1	wt	intact	no	+*	+*		1997	Takita et al. (1997)
53	48	NB1^	na	deleted	nd	nd	-	not Amp	1998	Easton et al. (1998)
54	49	NB2	wt	intact	nd	nd	-/+	Amp	1998	Easton et al. (1998)
55	50	NB3 ^^	wt	intact	nd	nd	-/+	not Amp	1998	Easton et al. (1998)
56	X	NB4	wt	intact	nd	nd	+++	Amp	1998	Easton et al. (1998)
57	X	NB5	wt	intact	nd	nd	+++	Amp	1998	Easton et al. (1998)
58	51	NB6	wt	intact	nd	nd	++	Amp	1998	Easton et al. (1998)
59	52	NB8	wt	intact	nd	nd	++	Amp	1998	Easton et al. (1998)
60	53	NB10	na	deleted	nd	nd	-	Amp	1998	Easton et al. (1998)
61	54	NB12	wt	intact	nd	nd	++	not Amp	1998	Easton et al. (1998)
62	55	NB13	wt	intact	nd	nd	+++	Amp	1998	Easton et al. (1998)
63	X	NB14**	wt	intact	nd	nd	++	Amp	1998	Easton et al. (1998)
64	56	NB16^	wt	intact	nd	nd	+	not Amp	1998	Easton et al. (1998)
65	X	NB17	wt	intact	nd	nd	+++	Amp	1998	Easton et al. (1998)
66	57	NB19^	wt	intact	nd	nd	+++	Amp	1998	Easton et al. (1998)
67	X	NB20	wt	intact	nd	nd	++	Amp	1998	Easton et al. (1998)
68	58	NB21‡‡	wt	intact	nd	nd	++++	not Amp	1998	Easton et al. (1998)
69	59	CHLA-10	nd	intact	nd	nd	nd	not Amp	2001	Thompson et al. (2001)
70	60	CHLA-42	nd	intact	nd	nd	nd	not Amp	2001	Thompson et al. (2001)
71	61	CHLA-51	nd	intact	nd	nd	nd	not Amp	2001	Thompson et al. (2001)
72	62	CHLA-52	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
73	63	CHLA-54	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
74	64	CHLA-60	nd	intact	nd	nd	nd	not Amp	2001	Thompson et al. (2001)
75	65	CHLA-79	nd	intact	nd	nd	nd	not Amp	2001	Thompson et al. (2001)
76	66	CHLA-90	nd	intact	nd	nd	nd	not Amp	2001	Thompson et al. (2001)
77	67	CHLA-95	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
78	68	CHLA-98	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)

79	69	CHLA-101	nd	deleted	nd	nd	nd	Amp	2001	Thompson et al. (2001)
80	70	CHLA-103	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
81	71	CHLA-108	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
82	72	CHLA-124	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
83	73	CHLA-132	nd	intact	nd	nd	nd	not Amp	2001	Thompson et al. (2001)
84	74	CHLA-136	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
85	75	CHLA-138	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
86	76	CHLA-140	nd	intact	nd	nd	nd	not Amp	2001	Thompson et al. (2001)
87	77	CHLA-143	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
88	78	CHLA-150	nd	intact	nd	nd	nd	not Amp	2001	Thompson et al. (2001)
89	79	CHLA-152	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
90	80	CHLA-153	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
91	81	CHLA-171	nd	intact	nd	nd	nd	not Amp	2001	Thompson et al. (2001)
92	82	CHLA-174	nd	deleted	nd	nd	nd	not Amp	2001	Thompson et al. (2001)
93	83	CHLA-178	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
94	84	CHLA-179	nd	deleted	nd	nd	nd	Amp	2001	Thompson et al. (2001)
95	85	CHLA-185	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
96	X	CHP-134	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
97	86	CHP-901	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
98	87	CHP902R	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
99	X	LAN-5	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
100	88	LAN-6	nd	deleted	nd	nd	nd	not Amp	2001	Thompson et al. (2001)
101	89	N206	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
102	X	NB-69	nd	intact	nd	nd	nd	not Amp	2001	Thompson et al. (2001)
103	X	NBL-S	nd	intact	nd	nd	nd	not Amp	2001	Thompson et al. (2001)
104	X	NGP	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
105	X	NLF	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
106	90	NMB	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
107	91	SK-N-AS	nd	intact	nd	nd	nd	not Amp	2001	Thompson et al. (2001)
108	92	SK-N-BE	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
109	93	SK-N-F1	nd	intact	nd	nd	nd	not Amp	2001	Thompson et al. (2001)
110	X	SK-N-SH	nd	intact	nd	nd	nd	not Amp	2001	Thompson et al. (2001)
111	X	SMS-KAN	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
112	94	SMS-MSN	nd	intact	nd	nd	nd	Amp	2001	Thompson et al. (2001)
113	95	BE(2)-M17	nd	intact	no	nd	nd		2002	Harada et al. (2002)
114	X	BE2C	nd	intact	no	nd	nd	Amp	2002	Harada et al. (2002)
115	96	CHP-212	nd	intact	no	nd	nd		2002	Harada et al. (2002)
116	X	IMR32	nd	intact	no	nd	nd	Amp	2002	Harada et al. (2002)
117	97	SH-SY5Y	nd	intact	no	nd	nd		2002	Harada et al. (2002)
118	X	SK-N-AS	nd	intact	no	nd	nd	not Amp	2002	Harada et al. (2002)
119	X	SK-N-BE	nd	intact	no	nd	nd	Amp	2002	Harada et al. (2002)
120	98	SK-N-DZ	nd	intact	no	nd	nd		2002	Harada et al. (2002)
121	X	SK-N-F1	nd	intact	no	nd	nd	not Amp	2002	Harada et al. (2002)
122	X	SK-N-MC	nd	intact	no	nd	nd	not Amp	2002	Harada et al. (2002)
123	X	SK-N-SH	nd	intact	no	nd	nd	not Amp	2002	Harada et al. (2002)
124	X	IMR32	nd	intact	no	no	no	Amp	2003	Obana et al. (2003)
125	X	LAN-2	nd	intact	yes	no	no		2003	Obana et al. (2003)

Table 1: *P16* gene and expression data in neuroblastoma cell lines. Summary of the literature of *p16* status in neuroblastoma cell lines. A total of 125 analyses of 98 unique neuroblastoma cell lines were identified revealing 7 deletions (7%).
‡C A mutation two base pairs upstream of the *p16* translation start site; *Extent of expression not quantified; ^ It is believed these are unique cell lines with overlapping names; ^^atypical neuroblastoma, see (Easton et al., 1998); **CDK6 mutation. However, appears to be cell culture acquired as we could not confirm this mutation in our study (Diccianni et al., 1999). ‡‡ *CDK4* amplified.

time a complete compilation of all studies through 2005 in which the *p16* gene was investigated in neuroblastoma cell lines. It reveals that out of 125 neuroblastoma cell lines analyzed, 98 of which were non-overlapping, only 7 (7%) harbored *p16* homozygous deletion.

Promoter hypermethylation is a common alternative mechanism of *p16* and *p15* gene inactivation in many tumor types (Baylin et al., 1998). In neuroblastoma, however, we found no evidence of *p16* hypermethylation in 14 *p16*-intact neuroblastoma cell lines tested when we focused on the methylation-sensitive Eag I restriction site of *p16* exon 1 (Diccianni et al., 1996). A separate analysis of 11 neuroblastoma cell lines using methylation-specific PCR also failed to find any evidence of *p16* gene methylation in this same region (Harada et al., 2002). In infrequent instances, however, methylation of the *p16* promoter region has been observed. Takita et al. (Takita et al., 1997) observed methylation at the *p16* exon 1 Sma I site in 5 of 19 neuroblastoma cell lines that correlated with loss of expression (Takita et al., 1998). In primary neuroblastoma, stage-independent *p16* methylation at adjacent *p16* exon 1 Sac II or Sma I sites was observed in several samples (Iolascon et al., 1998). Methylation did not correlate with *p16* expression in these samples and was never found at both sites at the same time, even though the restriction sites (along with Eag I) together span a single 15 base CpG-rich fragment of the *p16* gene. This indicates that the *p16* undergoes partial methylation that is likely insufficient to silence transcription in most cases.

Thus inactivation of the p16 gene by deletion, mutation, or promoter hypermethylation is an infrequent event in neuroblastoma and as such not likely involved in disease pathogenesis.

IV. P16 ACTIVATION IN NEUROBLASTOMA

Considering the infrequency with which molecular alterations of *p16* were found in neuroblastoma cell lines and primary tumor samples, it seemed to us at the time a forgone conclusion that there would be no role for p16 in neuroblastoma pathophysiology. However, considering that *p27* harbors infrequent molecular alterations yet is prognostically significant in many cancers due to its post-transcriptional down regulation, we felt it was important to document *p16 expression* levels in neuroblastoma. We had observed that normal foreskin fibroblasts expressed low but easily detectable levels of p16 protein and transcript using immunohistochemistry, western blot and semi-quantitative PCR, while the pRb-inactivated osteosarcoma cell lines Saos2 expressed very high levels of p16 protein and transcript. When we examined the expression profiles of p16 in neuroblastoma cell lines, we observed that about one third of the samples expressed very high levels of p16 that were comparable to those seen in the Saos2 cell line (Diccianni et al., 1999). Furthermore, in contrast with the known role of p16 as an inhibitor of pRb phosphorylation, the pRb protein was present and hyperphosphorylated in all neuroblastoma cell lines independently of p16 expression status. Notably, both p16 expressing and non-expressing neuroblastoma cell lines proliferated at similar rates, suggesting a failure of p16 to inhibit cell cycle arrest (unpublished data).

To explain the paradoxical expression of p16 in neuroblastoma, we opted to undertake a comprehensive expression analysis of the G1 cell cycle regulatory pathway to identify deregulatory steps which may functionally negate p16 activity (Diccianni et al., 1999). Two p16-expressing cell lines and one primary neuroblastoma sample was amplified for *CDK4*, offering a mechanism of cell cycle deregulation and p16 "resistance" for these samples. No deregulations of other components of the cell cycle, including *CDK6* or *cyclin D1*, were observed in any other cell line. *Cyclin D2* was infrequently expressed in neuroblastoma and not correlated with p16 expression. As has been observed in breast cancer, multiple isoforms of cyclin E are present in neuroblastoma cell lines, though again no correlation with p16, pRb or proliferation was observed. Finally, pRb regulated transcription factors E_2F1 and E_2F2 were expressed at comparable levels in neuroblastoma cell lines. This data suggest that expression of p16 bypasses the normal regulatory control elements in neuroblastoma, and may be indicative of deregulatory events outside of the normal cell cycle mechanisms.

The observation that neuroblastoma cell lines express high levels of p16 was also observed by Easton at al. (Easton et al., 1998). In addition to p16 expression, these investigators observe a high level of cyclin D/CDK6 protein kinase activity and a functional pRb, each of which should be inhibited by p16. The paradox of high p16 expression could be explained in the NB14 neuroblastoma cell line (Table 1) by the finding of a *CDK6* mutation that results in constitutive activation of the protein. However, we were unable to confirm this *CDK6* mutation in this or any neuroblastoma cell line, suggesting the mutation was acquired in cell culture (unpublished data; (Diccianni et al., 1999). We further failed to identify any mutations in the active site of *CDK4*, where activating mutations which can negate the influence of p16 have been observed (Diccianni et al., 1999). The observation that neuroblastoma cell lines express high levels of p16 was also observed but underappreciated by Takita et al., where 6 cell lines highly expressed *p16* transcript, with two highly expressing p16 protein (Takita et al., 1998).

P16 expression in neuroblastoma is not restricted to cell lines. In an analysis of primary neuroblastoma, we observed that p16 transcript and protein was significantly more frequently expressed in advanced stage neuroblastoma (stage 3 and 4) than in favorable stage neuroblastoma (stage 1, 2 and 4s). As in cell lines, deregulatory events of the G1 regulatory pathway downstream of p16 (cyclins, CDKs and pRb) were rare and not correlated with p16 expression. Expression of p16 was also prognostically significant, as *p16* expression was significantly associated with a lower overall survival (Omura-Minamisawa et al., 2001). These findings contrast with the report suggesting the *lack* of p16 expression significantly correlated with the unfavorable stage of the disease (Takita et al., 1998). However, several design flaws in the latter study results in a sample population that is poorly representative of the overall neuroblastoma population. The proportion of patients >1 year of age and of *MYCN* amplification, both of which are well-known adverse prognostic factors, is lower in the Takita study than in our reports (age >1 year, 38% in Takita's study *versus* 60% in ours; *MYCN* amplification, 9% *versus* 20%, respectively). Overall, the patient population in Takita's study appears to represent lower risk neuroblastomas compared with that in our study, where the stage (risk) distribution is fairly representative of the distribution of stages across the general population of patients with neuroblastoma. At least two other studies investigated *p16* expression in primary neuroblastoma. Iolascon *et al.* (1998) show stage independent *p16* transcript expression in 50% of the primary neuroblastoma investigated (Iolascon et al., 1998), while Obana et al. (2003) demonstrate *p16* expression in 84% of the primary neuroblastoma investigated (Obana et al., 2003). However, it is unclear in both these studies what the expression of p16 is relative to, so it is unclear if this is "normal", high or low *p16* expression.

The activation of *p16* expression, its lack of cell cycle inhibitor function, and the absence of alterations in the normal cell cycle regulatory machinery suggests normal regulatory mechanisms have been bypassed in neuroblastoma.

V. ID2 AND P16 EXPRESSION IN NEUROBLASTOMA

It is well documented that when pRb is deleted, inactivated or hyperphosphorylated, *p16* transcription is stimulated. Id2 is a helix–loop–helix (HLH) protein that has been shown to influence cell cycle progression through its ability to bind and inactivate pRb without an influence on pRb phosphorylation (Iavarone et al., 1994; Lasorella et al., 1996). We hypothesized that inactivation of pRb by Id2 might drive the paradoxical *p16* expression independently of pRb phosphorylation status. Furthermore, as pRb is inactivated, the expression of p16 would have no effect on cell growth. However, our investigations revealed that all neuroblastoma cell lines expressed Id2 at comparable levels, even though only approximately one-third of the cell lines expressed *p16* transcript and protein (Gebauer et al., 2004). The degree of Id2 expression was further not related to *p16* expression, with most neuroblastoma cell lines expressing Id2 at levels analogous to those found in differentiating cells. An analysis of primary neuroblastoma samples further confirmed that this was not a cell line phenomenon as *Id2* transcript expression was observed in most neuroblastoma samples and was independent of *p16* expression. Thus the paradoxical expression of p16 in neuroblastoma cannot be explained by Id2 expression.

It is worth commenting at this point on the role of Id2 in neuroblastoma independently of p16. It has been widely reported that the overexpression of Id2 may be involved in neuroblastoma pathophysiology. *Id2* has been reported to be transactivated by *MYCN* (Lasorella et al., 2000), and correlate with *MYCN* amplification, neuroblastoma stage and a poor prognosis (Lasorella et al., 2002). However, this relationship appears to be suspect. Instead of *Id2* expression being associated with *MYCN* or prognosis, we observed that most neuroblastoma expressed *Id2* transcript in a stage-independent fashion. Furthermore, Id2 protein was undetectable or just barely detectable in most samples by western blot, regardless of *Id2* transcript expression status, and independent of neuroblastoma stage (Gebauer et al., 2002; Gebauer et al., 2004). A similar lack of relationship of Id2 expression and neuroblastoma prognosis has been reported by at least 5 other groups by different methods (Alaminos et al., 2005; Korja et al., 2005; Sato et al., 2003; Vandesompele et al., 2003; Wang et al., 2003). Each of these investigators, ourselves included, fail to demonstrate a relationship of *Id2* expression with *MYCN* amplification or expression.

Taken together, these results rule out a role for Id2 in neuroblastoma pathogenesis, *MYCN* function or *p16* expression.

VI. OTHER CDKIS IN NEUROBLASTOMA: P27 AND P18

P27 AND NEUROBLASTOMA

Molecular alterations of the *p27* gene, including deletions, mutations and promoter hypermethylation, are rare in most cancers, including neuroblastoma (Kawamata et al., 1996; Sgambato et al., 2000; Viglietto & Fusco, 2002). However, post-translational inactivation of p27 is common and prognostically significant in many cancers (Philipp-Staheli

et al., 2001). P27 may be involved in neuroblastoma pathogenesis as well. Bergmann et al. reported that patients harboring p27-postive tumors had a significantly longer survival than p27-negative tumors and that p27 was an independent prognostic factor (Bergmann et al., 2001). In neuroblastoma cell lines, we have observed that p27 protein is expressed very highly in some cell lines, and at very low levels in others (Gebauer et al., 2004). This observation proved highly significant in primary neuroblastoma, where p27 protein was expressed in significantly fewer unfavorable stage neuroblastomas than in favorable stage protein, supporting a pathogenetic role for p27 in neuroblastoma tumorigenesis.

In light of the complementary role p27 plays in facilitating p16-induced G1 arrest (see section II) and the potential for a prognostically significant role in neuroblastoma, we considered a role for p27 in the paradoxical expression of *p16* in neuroblastoma, with the loss of p27 circumventing the increase in *p16* expression. However, while high stage neuroblastomas have a significantly higher frequency of *p16* expression and a significantly lower frequency of p27 expression, an inverse relationship between the two proteins was not observed (Gebauer et al., 2004).

Thus while decreased expression of p27 is associated with a poor prognosis in neuroblastoma, the paradoxical expression of *p16* in neuroblastoma cannot be explained by loss of p27 protein.

P18 AND NEUROBLASTOMA

The *p18* gene lies on chromosome 1p32 and shares a high degree of functional and sequence homology with *p16*, though p18 preferentially inhibits CDK6, while p16 inhibits both CDK4 and CDK6 equally (Guan et al., 1994). It is known that the short arm of chromosome 1p is the most frequently altered chromosomal segment in neuroblastoma, with alterations encompassing mostly deletions (Brodeur et al., 1977; Schleiermacher et al., 1994). As many as 70-80% of the neuroblastoma cell lines and primary tumors harbor 1p deletions in the region of 1p30 to 1p36, with at least one primary target of deletion residing at 1p32 (Brodeur & Fong, 1989). However, despite the observation that *p18* localizes to 1p32, molecular alterations of *p18* have not been found in neuroblastoma (Diccianni et al., 1996; Easton et al., 1998; Kawamata et al., 1996), suggesting it is not the target of inactivation at this locus.

This data suggests that deletions of 1p in neuroblastoma are not prognostically significant in neuroblastoma due to the inactivation of p18.

VII. CONCLUSIONS

Inactivation of *p16* has been observed in virtually every tumor type investigated, neuroblastoma being a major exception. In contrast to inactivation, *p16* is paradoxically highly expressed in many neuroblastoma cell lines and primary tumor, with expression associating with a poorer outcome. As infrequent alterations of the downstream pathway to p16 were observed, it remains an enigma as to not only why p16 is so highly expressed in neuroblastoma, but why it fails to arrest the cell cycle. Despite our greater understanding of the molecular mechanisms underlying adrenal cell function and phenotype, the role of p16, if any, in neuroblastoma has yet to be determined. The answer to this question, we believe, will shed light on the deregulation of growth arrest that exists in neuroblastoma, and may shed light on directions for therapeutic intervention. Furthermore, we believe that the molecular profiling of the genetic changes and expression patterns of neuroblastoma could lead to an even more precise sub-classification system that will be predictive of outcome, as well as therapies to which the tumor is most likely to be responsive.

ACKNOWLEDGEMENTS

I thank Dr. R. Chilcote for critical reading of this manuscript, Dr. A. Yu for many years of an ongoing and highly productive research partnership, and Mr. Greg Best for administrative assistance. Supported in part by an American Cancer Society/ Institutional Research Grant #IRG-70-002-29, the Moores UCSD Cancer Center, the San Diego Padres Cindy Matters Foundation (MBD), the Children's Oncology Group (COG) and Grant MO1-RR00827 from the General Clinical Research Center program of the National Center for Research Resources, NIH.

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