CORRELATION OF PCNA EXPRESSION WITH CLINICO-PATHOLOGIC FEATURES OF NEUROBLASTOMA

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INTRODUCTION AND AIM OF THE STUDY: Neuroblastoma, a tumor arising from the sympathetic nervous system, is one of the most common childhood tumors. Among the clinical factors with prognostic value, age at diagnosis and stage have been shown to be the most reliable. At the molecular level, a) *MYCN* oncogene amplification correlates with advanced disease stage, b) loss of heterozygosity studies show that chromosome arms 1p and 14q independently suffer losses of genetic material in neuroblastoma, and c) the expression of *HRAS* , *SRC, TRK* and *LNGFR* oncogenes correlate significantly with a better overall prognosis in neuroblastoma patients.

Tumor suppressor genes and molecules involved in the control of the cell cycle are presently being tested to elucidate whether they are involved in neuroblastoma tumorigenesis. The p53 tumor suppressor gene is the most commonly mutated gene in human cancer. Proliferating cell nuclear antigen (PCNA) is a 36kD cell-cycle-related nuclear protein that is maximally elevated in late G1 and S phase of proliferating cells. The aim of this study was to determine the mutation pattern of the p53 tumor suppressor gene and PCNA expression in 29 neuroblastic tumors, in order to make correlations with known clinicopathologic features.

MATERIAL AND METHODS: A series of 29 neuroblastic tumors from Hospital La Paz was studied. After surgical removal, the tumors were excised into two parts, one was fixed in 10% formalin for 24 h and embedded in paraffin for pathological study, and the other one, snap frozen in liquid nitrogen and stored at -70ºC for molecular analysis. Tumors were classified according to their localisation, pathologic diagnosis, clinical stage, stroma content, and neuroblastic differentiation (Table 1).

Detection of p53 mutations: After extraction of DNA from fresh-frozen tumors, we amplified exons 5 through 8 of the p53 gene by means of the polymerase chain reaction technique (PCR) and then we subjected those amplified radioactively labelled DNAs to single-strand conformation polymorphism (SSCP) analysis in 6% polyacrylamide (20:1 acrylamide:bis-acrylamide) and 4.5% polyacrylamide (49:1 acrylamide:bis-acrylamide) nondenaturing gels with or without 10% glycerol. Gels were run at 3-5 W for 18-24 h at room temperature (with glycerol) and at 4ºC in a cold room without glycerol), dried at 80ºC for 2 h, and exposed to X-ray films with intensifying screens at -70ºC between one and 2 days.

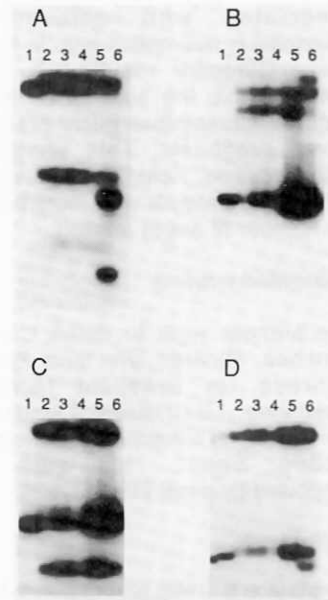

Figure 1. p53 mutation detection by PCR-SSCP in three neuroblastomas (2,3,4). Controls: non-denatured DNA (1), blood DNA (5), and positive controls (6). Exons 5 to 8 (A to D) of the p53 gene were studied

p53 and PCNA immunohistochemical expression: Sections were cut from the paraffin blocks, and routine immunohistochemical methods were used to detect p53 and PCNA proteins. Mouse monoclonal primary antibodies for p53 (Oncogene Research Products) and PCNA (Biomeda) were used at dilutions of 1:20 and 1:400 respectively. Sections were incubated with biotynilated goat antimouse IgG (Dako) as the secondary antibody, at a dilution of 1:400. Signal was visualized using the streptavidin-biotin-peroxidase method. The slides were counter stained with methyl green.

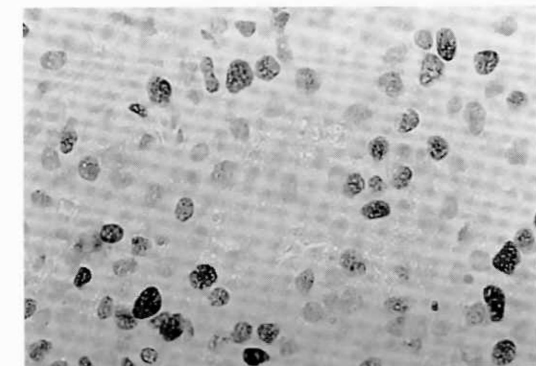

Figure 2. Immunohistochemical detection of PCNA in neuroblastoma. Diffuse pattern of immunostaining. (X500).

Statistical analysis: The logistic regression test was used to study the correlation between the presence of immunoreactivity for PCNA and the following variables: tumor localisation, anatomopathologic diagnosis, clinical stage, stroma abundance, degree of differentiation, and presence of cytoplasmic p53 protein after immunohistochemistry. The McNemar test was also included for the valuation of the dependence between PCNA immunoreactivity and stroma abundance, degree of differentiation, and presence of cytoplasmic p53 protein after immunohistochemistry. In order to facilitate the calculations, PCNA expression was divided in positive (focal and diffuse) and negative (isolated and negative)

RESULTS AND CONCLUSIONS: We could not detect any mobility shift (mutation) in our SSCP experiments. On the contrary, p53 immunohistochemical expression in the same cases gave as a positive result in 22 cases, 2 of which corresponded to nuclear staining, and 20 to cytoplasmic staining. According to our results, p53 accumulated protein corresponds to the wild type, as no mutations were found by the molecular techniques. Wild-type p53 protein has been found to be abnormally sequestered in the cytoplasm of primary human tumors, including neuroblastoma. This may represent a non-mutational

mechanism for abrogating p53 tumor suppressor function in neuroblastoma.

PCNA expression was detected and classified as indicated in Table 1. The statistical analysis revealed a significant association between PCNA immunostaining and location of the tumor, stroma abundance and degree of differentiation. In this sense, neuroblastomas located in the adrenal gland, and the stroma poor group of neuroblastic tumors expressed PCNA more frequently. Surprisingly, we also found an statistical association between the differentiating type of neuroblastomas and PCNA expression.

Although this is mathematically acceptable, it is also true that diagnosing the degree of differentiation of the tumor is rather subjective while defining the stroma content of the tumor is more definite. PCNA expression has been associated with unfavourable prognosis in neuroblastoma. In light of our preliminary results we might conclude that the adrenal or stroma poor neuroblastomas might present a worse prognosis. This should be further studied, including the follow up data of the patients and incrementing the number of cases studied.

TABLE 1		% CASES WITH PCNA IMMUNOREACTIVITY			
		Negative	Isolated	Focal	Diffuse
LOC(#cases)	Adrenal (12)	8.3	8.3	16.6	16.6
	Mediastine (4)	50	25	-	25
	Soft tissues(7)	43	-	-	57
	Retroperitoneum (2)	-	50	-	50
	Others (4)	75	-	-	25
APD(#cases)	Neuroblastoma(18)	16.6	11.1	11.1	61.1
	Ganglioneurobl(6)	50	16.6	-	33.3
	Ganglioneuroma(5)	60	-	-	40
CLINICAL STAGE (#cases)	I (4)	-	-	25	75
	II-A (0)	-	-	-	-
	II-B (2)	-	50	-	50
	III (4)	25	25	-	50
	IV (9)	22.2	11.1	11.1	55.5
	IV-S (1)	-	-	-	100
STROMA (#cases)	Poor (15)	6.6	13.3	13.3	66.6
	Rich (7)	28.5	-	-	71.4
DIF(#cases)	Differentiating (7)	-	14.2	-	85.7
	Undifferentiated (10)	10	10	20	60
p53nuc (#cases)	Yes (2)	-	-	-	100
	No (27)	33.3	11.1	7.4	48.1
p53cyt (#cases)	Yes (20)	30	10	5	55
	No (9)	33.3	11	11	44.4

Key. LOC: localization, APD: anatomopathologic diagnosis, DIF: degree of differentiation, p53nuc: nuclear immunoreactivity for p53 protein, p53cyt: cytoplasmic immunoreactivity for p53 protein.

Assessment of PCNA immunoreactivity. Negative: no positive nuclei. Isolated: one or few scattered positive nuclei, without any clusters. Focal: positive nuclei aggregated in focal area (s). Diffuse: positive nuclei distributed homogeneously.

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