

Apoptosis in peripheral neuroblastic tumors. Immunohistochemical expression of bcl-2 and p53 is related to DNA fragmentation

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Summary. We examined 111 cases of neuroblastoma (NB), searching for how NB relates to apoptotic control and other prognostic factors. Immunohistochemistry using avidin-biotin-peroxidase was carried out for bcl-2 and p53 proteins. Apoptosis was analyzed by *in situ* detection of chromosomal breakdown. DNA ladders were detected by electrophoresis and amplification of *MYCN* was carried out by PCR and Southern blot. Statistical analyses were performed with Pearson's χ^2 and Kruskal-Wallis tests and Cox's regression. We found expression of bcl-2 protein mainly in cases of neuroblastoma without differentiation and in stages 3 and 4. Expression of p53 protein showed a correlation with bcl-2 and the apoptotic phenomenon; apoptosis was found mainly in favorable cases. Multivariate analysis showed bcl-2 protein expression to be the most independent risk factor. The study of apoptosis could be important for the design of therapies to treat neuroblastoma.

Key words: Neuroblastoma, Biochemical apoptosis, Apoptosis *in situ*

Introduction

Peripheral neuroblastic tumors (PNT) are a heterogeneous group of neoplasms that includes different categories of neuroblastoma (NB), ganglioneuroblastoma (GNB), and ganglioneuroma (GN). All of them present markedly divergent clinical and biological factors of prognostic relevance, such as the age of the patient, tumoral staging, *MYCN* amplification, and deletion of the 1p chromosome.

It is widely accepted that some favorable

neuroblastomas regress spontaneously, and maturation to ganglioneuroma occurs in others (Matsumura et al., 1991); probably, PNT are tumors in which mutated cells have abnormalities in programmed cell death. Cells undergoing programmed cell death display distinctive morphological changes including rapid blebbing of the plasma membrane and nuclear disintegration. Nuclear collapse is preceded by damage of the chromatin, which has been recognized as a DNA ladder easily observable by agarose gel electrophoresis. One of the most studied characteristics of apoptosis is the DNA fragmentation (Cohen et al., 1993). A method to detect the apoptosis process *in situ* uses terminal deoxynucleotidyl transferase to incorporate a biotinylated derivative of dUTP' at the new 3'-OH termini produced in the DNA by the endonucleotic activity associated with chromosomal breakdown (Gavrieli et al., 1992; Gorczyca et al., 1993).

Apoptosis is a complex route regulated by several oncogenes and tumor suppressor genes. In this process, bcl-2 and p53 play important roles. There is some controversy about possible relationships between overexpression of bcl-protein, *MYCN* amplification, deletion of chromosome 1p, histoprognosis, and/or clinical staging (Schwab et al., 1994; Schleiermacher et al., 1994).

Although bcl-2 has been found in differentiated tumors such as ganglioneuromas (Look et al., 1991), the results of the other reports indicate that high expression of bcl-2 protein could indicate a poor prognosis in NB. We have previously described a close correlation between bcl-2 protein expression and apoptotic inhibition in NB (Mejía et al., 2002). Another important factor that participates in the control of apoptosis is p53 activation of bax expression, which in turn blocks the ability of bcl-2 to inhibit apoptosis.

The main purpose of this paper is to demonstrate that the apoptotic phenomenon could be studied even in archived material and how it relates to other well-known prognostic indicators in neuroblastoma.

Materials and methods

Samples

We studied 111 cases of PNT from the files of the Spanish Neuroblastoma Registry (Spanish Cooperative Protocol N-II-92) (Castel et al., 1995) prior to chemotherapy. Each case was staged according to the INSS classification (Brodeur et al., 1988) and histopathologically classified as favorable or unfavorable according to INPC (Shimada System) (Peuchmaur et al., 2003; Navarro et al., 2006). We studied a total of 83 NB, 18 GN, and 10 GNB (6 diffuse, 2 NOS and 2 nodular).

Immunohistochemical analysis

Paraffin-embedded tumors were cut in 5 μm sections and analyzed by immunohistochemistry for bcl-2 (IgG1 clone 124, DAKO; dilution 1:50) and p53 (DO7, DAKO; dilution 1:10) using the avidin-biotin-peroxidase (ABC peroxidase) method (Hsu et al., 1981). A microwave oven treatment with target unmasking fluid (TUF) (citrate buffer pH 6) was performed (Cattoretti et al., 1993). The color was developed using 0.02%, 3-3'-diaminobenzidine (DAB) tetrachloride and 0.02% hydrogen peroxide in Tris-HCl buffer (pH 7.4), and then sections were counterstained with Harris' hematoxylin.

In situ assay for apoptosis

This analysis was carried out in 111 NBs by an *in situ* detection method (ApopDETEK, ENZO Diagnostics, Inc., USA), according to the manufacturer's instructions. Briefly, paraffin-embedded tumoral sections of 5 μm were permeabilized with proteinase K 1x and incubated with terminal deoxynucleotide transferase (TDT) to incorporate Bio-16-dUTP at 3'-OH termini of the DNA. Incorporation was visualized using avidin-peroxidase followed by DAB and counterstained with Harris' hematoxylin. In addition, in 96 samples of NB, genomic DNA was isolated from frozen tissue by phenol/chloroform extraction, treated with proteinase K (Sambrook et al., 1989), and then electrophoresed in agarose gels (1%).

MYCN study

DNA was isolated as previously described (Sambrook et al., 1989). A differential PCR to screen for *MYCN* oncogene amplification was performed (Crabbe et al., 1992). Results were confirmed by Southern blot; 10 μg of each sample was digested with *ECORI* or *HindIII*, then run in a 1% agarose gel, and transferred to Hybond-N (Amersham) membranes. For hybridization a 1-kb *MYCN* probe (ONCOR, USA) was used.

Statistical analysis

To assess the significance of the association of the

variables studied, we calculated Pearson's χ^2 and used the Kruskal-Wallis test (SPSS program, Norusis 1994). Survival analysis was performed with Kaplan Meier test analysis, and the correlations were assessed with Cox's regression.

Tumoral reactivity

The immunohistochemical profile was classified as negative expression (-) when there were less than 5% positive cells, whereas all other cases were considered positive (+).

Results

Survival analysis

In our series, the average age of patients with NB was 2.12 years with a predominance of male over female patients in children older than 1 year.

Morphological identification of apoptotic cells in NB

According to the International Neuroblastoma Pathology Classification (INPC), a great number of tumors were considered favorable NB (85/111). Individual cells presented hyperchromatic dense nuclei and scanty rim of cytoplasm. In some areas, the tumor cells had undergone "karyorrhexis", characterized by picnotic or smashed nuclei and eosinophilic cytoplasm. There were no inflammatory infiltrates around the karyorrhetic cells.

Bcl-2 expression

We found cytoplasmic expression of bcl-2 protein in 68 cases (Fig. 1A), which were mainly poorly differentiated NB. This marker was expressed in 31 apoptotic cases and in advanced stages of the disease. An association between bcl-2 protein expression and *MYCN* amplification was found in 8 of 11 cases; however, this relation was not significant ($p > 0.05$, Table 1). In general, the cases with bcl-2 expression had the worst survival rate ($p = 0.0397$).

p53 protein expression

Only 7 of 111 cases expressed p53 protein (Fig. 1B); it was found mainly in unfavorable NB without *MYCN* amplification. In our series, all cases of p53 were also positive for bcl 2; this relation was significant.

Detection of apoptosis

Examination of apoptosis in 111 cases by the *in situ* detection method showed that 66 tumors contained cells that incorporated biotinylated dUTP into their nuclei. The labeled nuclei comprised small rounded fragments that were scattered or occasionally gathered together (Fig. 1C). The proportion of labeled apoptotic cells

DNA fragmentation in neuroblastoma

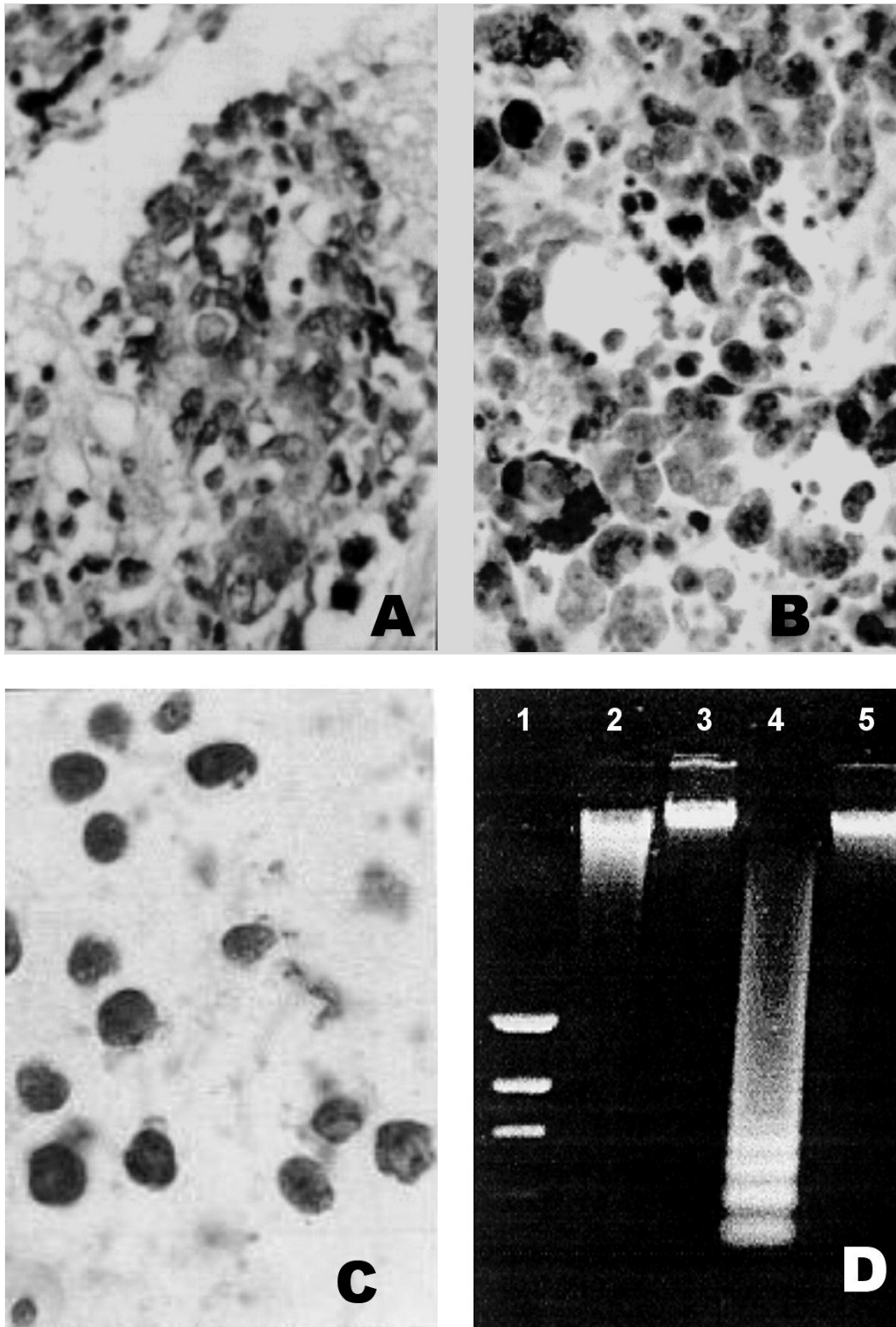
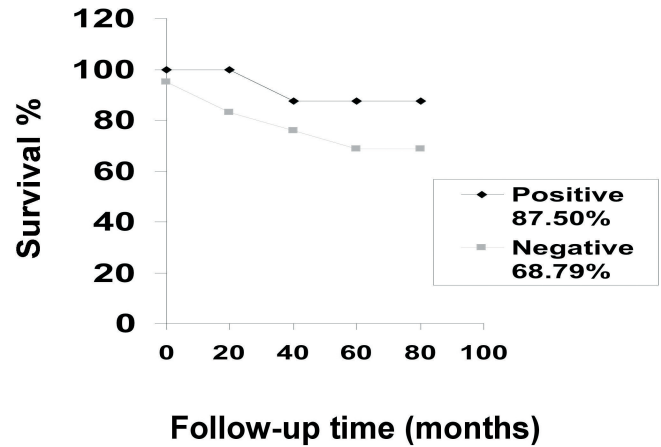
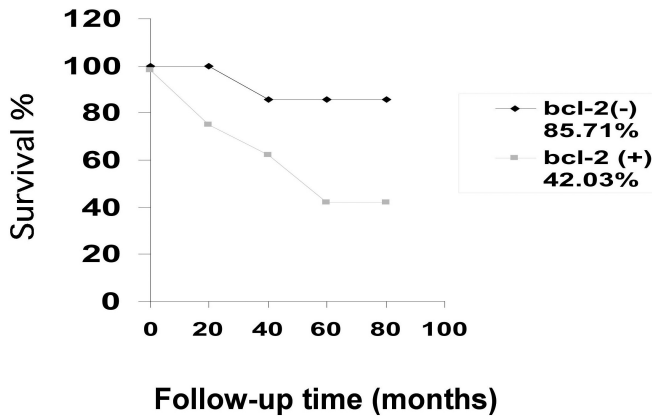


Fig. 1. Detection of molecules involved in apoptotic phenomenon. **A.** Bcl-2 expression in unfavorable, undifferentiated neuroblastoma (ABC peroxidase, x 40). **B.** Nuclear expression of p53 in a stroma-poor neuroblastoma (ABC peroxidase, x 40). **C.** *In vivo* detection of apoptotic nuclei in a stroma-rich neuroblastoma (ApopDETEK, x 100). **D.** Electrophoresis on 1% agarose gel showing a classic apoptotic ladder (lane 4) of fragmented DNA; line 1 marker of 100 bp; lines 2, 3 and 5: cases without apoptosis.

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ranged from 5% (+) to 100% (++++) of the tumor cells, and showed a significant correlation with biochemical detection of apoptosis ($p < 0.012$). We found that for these 66 patients, those without bcl-2 expression had a better survival rate (85.71%; Graph 1).

In 9 of 96 tumors, there was fragmentation of DNA into a ladder of regular subunits (180-200 base pairs) indicative of internucleosomal cleavage (Fig. 1D). Apoptosis was detected biochemically mainly in children younger than 1.5 years, without *MYCN*



Graph 1. Kaplan-Meier survival graphics correlating expression of apoptotic process. Survival in patients showing *in situ* apoptosis. Neuroblastoma patients without bcl-2 protein expression (n=35) presented a higher overall survival rate than the bcl-2 positive cases (n=31) in apoptosis detected by *in situ* assay.

Graph 2. Kaplan-Meier survival graphics correlating expression of apoptotic process. Biochemical assay for apoptosis. Survival was longer in NB patients with apoptosis (n=9) than without apoptosis (n=87), as detected by the biochemical method (87.50% vs 68.79%).

Table 1. Statistical relation between expression of bcl-2, p53, *in situ* apoptosis and biochemical apoptosis assays, and other factors related to apoptosis.

		BCL2		p	p53		p	In situ apoptosis		p	Biochemical apoptosis		p
		(-)	(+)		(-)	(+)		(-)	(+)		(-)	(+)	
Histology	NB	24	59		76	7		39	44		62	8	
	GNB	4	6	0.000 ^b	10	0	0.118 ^b	2	8	0.020 ^b	9	0	0.298 ^b
	GN	15	3		18	0		4	14		16	1	
Shimada	Fav.	37	48	0.048 ^a	82	3	0.030 ^b	33	52	0.661 ^b	67	8	0.414 ^b
	Unfav.	6	20		22	4		12	14		20	1	
Age	<1.5 years	15	33	0.174 ^a	47	1	0.112 ^b	21	27	0.685 ^b	35	7	0.032 ^b
	>1.5 years	28	35		57	6		24	39		52	2	
MYCN	Not amp.	39	58	0.407 ^b	92	5	0.098 ^b	40	57	0.756 ^b	75	9	0.279 ^b
	Amp.	3	8		9	2		4	7		12	0	
Stages	1-2	20	16		35	1		10	26		27	5	
	3-4	18	43	0.033 ^a	55	6	0.660 ^b	30	31	0.115 ^a	52	2	0.535 ^b
	4s	4	9		13	0		5	8		8	2	
Bcl-2	(-)	-	-	-	43	0	0.030 ^b	8	35	0.000 ^a	33	5	0.306 ^b
	(+)	-	-	-	61	7		37	31		54	4	
p53	(-)	43	61	0.030 ^b	-	-	-	39	65	0.012 ^b	81	8	0.645 ^b
	(+)	0	7		-	-	-	6	1		6	1	
In situ apoptosis	(-)	8	37	0.00 ^a	39	6	0.012 ^b	-	-	-	40	2	0.174 ^b
	(+)	35	31		65	1		-	-	-	47	7	
Biochemical apoptosis	(-)	33	54	0.306 ^b	81	6	0.645 ^b	40	47	0.174 ^b	-	-	-
	(+)	5	4		8	1		2	7		-	-	

Total: N=111 cases; Biochemical Apoptosis: n=96; MYCN: n=108 cases; Stages: n=110. ^a: χ^2 test; ^b: Kruskal-Wallis test. *: Statistical significance, $p < 0.05$. Fav=favorable, Unfav=unfavorable, Not amp=not amplified, Amp=amplified

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Table 2. Cox's regression.

Risk factor	Risk ratio	Confidence interval for risk ratio	p
Bcl-2	1.54	0.47-5.01	0.46
<i>In situ</i> assay	0.92	0.31-2.88	0.88
Biochemical Apoptosis	0.33	0.04-2.70	0.30
p53	0.26	0.07-0.98	0.04

*: Statistically significant, $p < 0.05$

amplification in tumors of stage 1 and 2, without p53 expression, and in cases with or without bcl-2 protein expression. However, none of these relationships were statistically significant ($p > 0.05$, Table 1). Overall survival was better in 9 patients with biochemically detected apoptosis than in those without (87.50% vs 68.79%; Graph 2).

Finally, as shown in Table 2, the multivariate analysis showed expression of the bcl-2 protein to be the only independent risk factor of our study (risk ratio 1.54).

Discussion

Our findings are in accord with previous reports (Castel et al., 1995; Saito et al., 1997) regarding the age of child survival, since the group younger than 1.5 years lived longer than the older patients.

We studied the apoptotic phenomenon from two points of view, analyzing the expression of the bcl-2 and p53 proteins and the apoptotic phenomenon *per se*. The physiological function of bcl-2 generally is different from the other oncogenes; its expression permits the cell to survive, due to its ability to avoid cell death by apoptosis (Hanada et al., 1993). At present, controversy exists regarding high levels of bcl-2 expression and how it relates to *MYCN* amplification, histopathology, and/or tumoral stage (Rudolph et al., 1993); therefore, we decided to measure bcl-2 expression in our samples.

We found that NB mainly unfavorable is positive for this marker. There are several reports correlating bcl-2 expression in NB with absence of morphological maturation. As well as others (Weinreb et al., 1995), we did not find a correlation between bcl-2 and *MYCN* amplification. Bissonette et al. (1992) found that over expression of the *c-myc* gene induced apoptosis in fibroblasts in conditions of starvation and absence of growth factor, a condition that does not necessarily exist *in vivo* in NB, and they concluded that *MYCN* amplification and bcl-2 expression could be independent processes in NB.

There was bcl-2 expression in cases with NB stages 3 and 4, as reported by Castle et al. (1990). We found a strong correlation between the presence of the bcl-2 molecule and the apoptotic phenomenon, suggesting that bcl-2 is linked to programmed cell death in NB.

It is very well known that p53 tumor suppressor

gene mutations are frequent in a wide variety of adult tumors (Nigro et al., 1989); however, in NB there are few reports on p53 expression (Hollstein et al., 1996). By immunohistochemistry we found only 7 cases of NB with p53 protein expression at the nuclear level (partial results were previously presented, Mejia et al., 1999). A frequent event in the origin and progression of many tumors is the loss of p53 gene function, which activates the apoptotic phenomenon. p53 can produce apoptosis by specific transcriptional transactivation, perhaps of bax, a member of the bcl-2 family that promotes apoptosis. In some cases, bax is not necessary for p53-dependent apoptosis, such as in γ -irradiated thymocytes (Knudson et al., 1995), where other factors may be more important; for example bcl-xL can inhibit p53-mediated apoptosis *in vitro*. We found only one p53-positive case with apoptosis; moreover, all 7 cases expressed bcl-2 protein. It is not clear if the apoptotic phenomenon in NB depends on another pathway different from p53. It has been demonstrated that some members of the bcl-2 family can inhibit p53-mediated apoptosis (Schott et al., 1995), but that this is not due to the inhibition of either p53 nuclear translocation or p53-mediated growth arrest. It seems that bcl-2 terminates the signal that activates the apoptotic machinery as a result of DNA damage.

There are a variety of protocols to detect apoptosis, but the *in situ* hybridization method has several advantages compared to the biochemical detection of apoptosis: since the break in DNA occurs before the changes in cell morphology, this assay can be used to study early events of apoptosis; it can be performed in NB embedded in paraffin (Mejia et al., 1998), and it can be performed by immunohistochemical analysis. Evidence of the apoptotic process was found in all tumoral types, but it was more frequently observed in GNB and GN, with more stage 4s cases showing apoptosis. It is imperative to confirm this fact, because one hypothesis for spontaneous regression of NB at stage 4s is that the apoptotic mechanism is working.

The proportion of risk was examined by a multivariate analysis using a Cox's regression. We found that PNT patients with bcl-2 expression have a poor outcome, as shown by the fact that bcl-2 expression was the most important risk factor for NB progression. The analysis of bcl-2 protein expression should be incorporated together with *MYCN* and 1p studies in the different models of therapeutic designs as a valuable and feasible prognostic indicator of PNT. Moreover, we recommend the *in situ* apoptosis assay over biochemical apoptotic detection of PNT, especially for retrospective studies.

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