Virchows Arch (1999) 434:301–305 © Springer-Verlag 1999

ORIGINAL ARTICLE

Chong Jai Kim · Je G. Chi · Hyung-Soo Choi
Hee Young Shin · Hyo Seop Ahn · Young Seok Yoo
Kwang-Yul Chang

Proliferation not apoptosis as a prognostic indicator in retinoblastoma

Received: 9 November 1998 / Accepted: 24 November 1998

Abstract The balance between proliferation and cell death is the major determinant of tumour growth. We analysed the proliferative and apoptotic indices (PI and AI, respectively) of 33 children with retinoblastoma. PI and AI were assessed by immunohistochemistry for Ki-67 antigen and TUNEL staining, respectively. The mean PI was 21.0±21.1%, and higher PI was associated with more advanced tumour stage ($P<0.0001$) and poor clinical outcome ($P<0.05$). Patients in whom amplified *N-myc* oncogene was found ($n=6$) determined by the multiplex polymerase chain reaction tended to have a higher PI (37.6±27.2%) than those without amplified *N-myc* ($n=27$; PI=17.3±18.1). A PI value of over 40% was clearly associated with an unfavourable prognosis. The AI, however, did not correlate with any of the other variables analysed. The findings suggest that proliferation, but not apoptosis, is of critical significance in retinoblastoma biology. PI, as determined by the Ki-67 antigen labelling index, seems to be a relevant histopathological parameter that can predict the clinical outcome of retinoblastoma.

Key words Proliferation · Apoptosis · Ki-67 antigen · Prognosis · Retinoblastoma

Introduction

Retinoblastoma is the most common ocular malignancy occurring in childhood, and since loss of retinoblastoma gene function is fundamental to tumour development, studies of this tumour have yielded important basic information on cancer genetics [4, 5, 11]. The tumour is believed to originate from cells that form the nucleated retina and is characterized histologically by the proliferation of small round cells with different spectra of differentiation and frequent coagulation-type necrosis with cuffs of viable cells [25]. Despite well-known clinicopathological characteristics, however, the histopathological variables that predict the clinical outcome of individual tumours are limited.

The growth of an individual tumour depends on an imbalance between proliferation and cell death; cell numbers are determined by an intricate balance of cell death and cell proliferation. The accumulation of cells through suppression of death or increased proliferation can contribute to both the development and progression of cancer [3]. These two variables have been frequently quoted as having prognostic significance in various human tumours [14, 23]. We evaluated the proliferative and apoptotic indices of retinoblastomas with special reference to their relationship with stage, amplification of *N-myc*, and clinical outcome. The proliferative index (PI), measured by the Ki-67 antigen-positive fraction, was significantly higher in advanced stage tumours, and patients with higher PI were characterized by poor clinical outcome. The apoptotic index (AI), however, did not correlate with all the factors analysed. This feature strongly suggests that, in retinoblastoma, it is proliferation and not apoptosis that is clinicopathologically significant, and that PI is relevant as a prognostic marker.

Materials and methods

A total of 33 cases of retinoblastoma were selected from the files of the Department of Pathology, Seoul National University Children's Hospital. All these patients had undergone enucleation, and serial 5-μm-thick sections were obtained from representative paraffin blocks. Clinical data were reviewed, and the patients were divided into two groups according to clinical outcome; those with no evidence of disease during follow-up were designated group I, while those who did have recurrent disease or who died were designated as group II. There were 20 boys and 13 girls. Five patients

C.J. Kim (✉)[1] · J.G. Chi · H-S. Choi · H.Y. Shin · H.S. Ahn · Y.S. Yoo
Departments of Pathology, Pediatrics, Ophthalmology,
Seoul National University College of Medicine, Seoul, Korea

K.-Y. Chang
Department of Ophthalmology, Catholic Medical School,
Seoul, Korea

Mailing address:
[1] Department of Pathology,
Seoul National University College of Medicine,
28 Yongon-dong, Chongno-gu, Seoul, 110-799, Korea
e-mail: cjkim@plaza.snu.ac.kr,
Tel.: 02-760-3549, Fax: 02-745-7966

Table 1 Profile of individual cases (*Age* months at diagnosis, *Lat* laterality, *U* unilateral, *B* bilateral, *PI* proliferative index, *AI* apoptotic index, *L* low, *H* high, *N-myc* N-myc amplification, *Stage* Reese-Ellsworth staging classification of retinoblastoma, *F/U* follow-up; *CVAM* cyclophosphamide+vincristine+doxorubicin+methotrexate, *CV* cyclophosphamide+vincristine, *CVM* cyclophosphamide+vincristine+methotrexate, *IEV* ifosfamide+etoposide+vincristine, * enucleation only, *NED* no evidence of disease, *D* died of disease, *R* recurrence)

Case	Age/sex	Lat	PI/AI	N-myc	Stage	F/U (months)	Treatment/ clinical outcome
1	15/F	B/F	0.98/L	–	1	29	CVAM, RT/NED
2	5/M	B/F	8.84/H	–	1	5	CVAM/D
3	22/M	B/F	4.38/L	+	1	60	CV, RT/NED
4	16/F	B/F	1.87/L	–	1	3	CVAM/D
5	37/F	B/F	34.71/L	–	1	66	CVAM/NED
6	21/F	U/S	69.82/H	–	4	10	IEV/R
7	68/F	U/S	21.02/L	–	4	17	CVAM, RT/D
8	18/F	U/S	31.37/L	–	3	19	CVAM/NED
9	25/M	U/S	6.34/H	–	2	6	CVAM/NED
10	30/M	U/S	31.27/H	–	2	49	CVAM, RT/R
11	46/F	U/S	8.52/L	–	4	93	CVAM/NED
12	7/M	U/S	18.95/L	–	3	92	CVAM/NED
13	12/F	U/S	5.83/L	–	2	7	CVAM/NED
14	10/F	U/S	61.56/L	+	4	20	CVAM, IEV/R
15	24/M	U/S	70.16/H	+	4	9	CVAM/NED
16	17/M	U/S	52.18/L	–	5	22	CVAM/D
17	79/M	U/S	33.21/L	+	4	57	CVAM/NED
18	23/M	U/S	41.67/L	–	3	23	CVAM/D
19	37/F	U/S	8.37/H	–	2	19	CVM/NED
20	99/F	U/S	9.14/L	–	2	66	CVAM/NED
21	41/M	U/S	8.65/L	+	1	17	*/NED
22	5/M	U/S	47.45/H	+	1	11	*/NED
23	81/M	U/S	7.67/H	–	1	12	*/NED
24	23/M	U/S	3.71/H	–	2	89	*/NED
25	90/M	U/S	13.45/L	–	1	7	*/NED
26	15/F	U/S	1.33/L	–	1	11	*/NED
27	7/M	U/S	11.20/L	–	2	40	*/NED
28	24/M	U/S	6.63/L	–	1	51	*/NED
29	26/M	U/S	2.14/H	–	1	54	*/NED
30	26/M	U/S	4.48/H	–	1	11	*/NED
31	76/M	U/S	9.83/H	–	1	88	*/NED
32	10/M	U/S	48.37/L	–	1	35	*/NED
33	26/F	U/S	7.72/H	–	1	71	*/NED

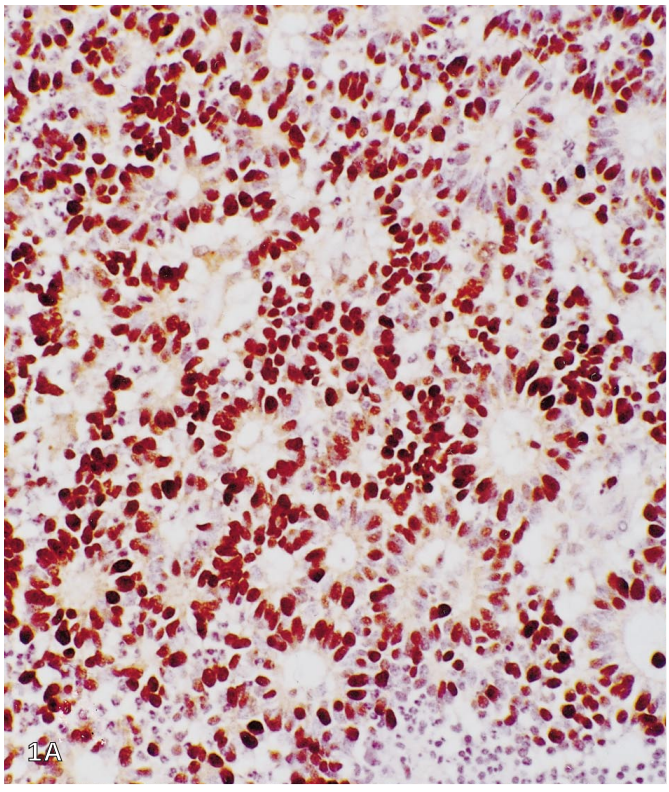
had bilateral tumours; these were all familial cases. Their age at diagnosis was 32.2±26.2 months, and the mean follow-up period was 35.7±29.0 months.

Sections were deparaffinized and hydrated through xylene and graded alcohol, and endogenous peroxidase was quenched with 3.0% hydrogen peroxide in methanol for 10 min. After PBS rinses, sections were incubated overnight at 4°C with 1:100 diluted murine anti-human Ki-67 antigen monoclonal antibody (Immunotech). For detection, a large volume Dako LSAB kit was used. Linking agent (biotinylated anti-rabbit and anti-mouse immunoglubulins in PBS) and streptavidin conjugated to horseradish peroxidase in Tris-HCl buffer were sequentially applied for 30 min each, with PBS rinses between the two steps. 3,3′-Diaminobenzidine was used as chromogen, and Meyer's haematoxylin for counterstaining. In each case, a minimum of 500 cells were counted and fraction (%) of the cells that showed positive nuclear staining for Ki-67 antigen was considered to be the PI.

For specific labelling of DNA fragmentation, an ApopTag in situ apoptosis detection kit was used (Oncor, Md.). The method used involved a minor modification of that described by Gavrieli et al. [6]. Deparaffinized sections were treated with proteinase K (20 µg/ml) for 15 min at room temperature, and after three rinses with PBS for 5 min each, 1× equilibration buffer was applied briefly. The sections were subsequently incubated at 37°C with a solution containing terminal deoxynucleotidyl transferase (TdT) and digoxigenin-labelled dUTP and dATP; the reaction was stopped by incubation for 30 min at 37°C in stop/wash buffer. After meticulous rinsing with PBS, drops of anti-digoxigenin-fluorescein were applied for 30 min at room temperature. Sections were observed under an immunofluorescence microscope (Zeiss, Germany); for positive controls sections were pretreated with DNase I (Boehringer Mannheim, Germany), and for negative controls TdT reaction solution was replaced by distilled water. In the solid area devoid of necrosis, apoptosis was evaluated at a magnification of ×250, and mean apoptotic counts were calculated from ten consecutive fields. The counts showed a bimodal frequency distribution of between 4 and 5/microscopic field; in cases with 5 or more apoptotic counts per field, AI was regarded as high; those with fewer than 5 per field were regarded as having low AI.

For retrospective analysis of *N-myc* amplification, DNA was extracted from 20-µm-thick serial sections using the conventional phenol–chloroform–proteinase K method. The primers used were 5′-TCACTGGGAGAAGCGGCGTT-3′ (5093–5113) and 5′-GTGCATCCTCACTCTCCACGT-3′ (5287–5267) for *N-myc*, and 5′-TCTTTTCTTTCCCGATAGGT-3′ (4582–4601) and 5′-CTGGGATGCTCTCTTCGACCTC-3′ (4731–4712) for γ-interferon. PCR was performed in standard buffer containing 1.5 mM of $MgCl_2$ in a total volume of 25 µl containing 10–20 ng of genomic DNA, 0.1 mM of *N-myc* primers, 0.5 mM of γ-interferon primers, 200 mM dNTP, and 1 U of Taq polymerase. The reaction was composed of 39 cycles of 1 min at 94°C, 1 min at 53°C, and 1 min at 72°C following initial denaturation at 94°C for 5 min. The amplified products (*N-myc*: 194 bp, γ-interferon: 149 bp) were compared on 2.5% agarose gel.

The relationship between PI, AI, *N-myc* amplification, stage, and survival was analysed using Cox's proportional hazards model. Cumulative survival was calculated using the Kaplan-Meier method, and log-rank analysis was used to determine the relationship between survival and PI. The relationship between *N-myc* amplification and PI was analysed by a *t*-test, and that between *N-myc* amplification and AI by a Chi-square test. The relationship between stage and PI, and stage and AI, was analysed by linear regression analysis and the Mantel-Haenszel Chi-square test, respectively.



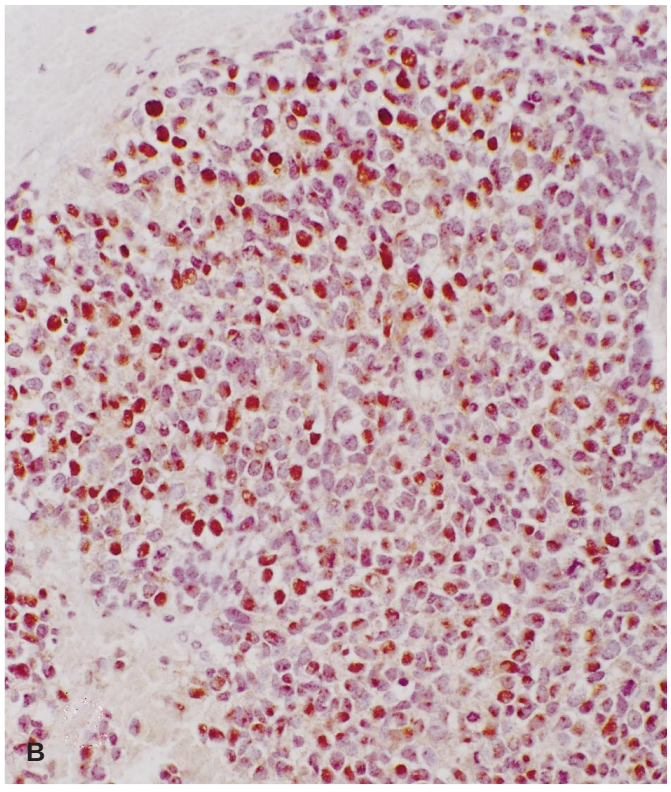
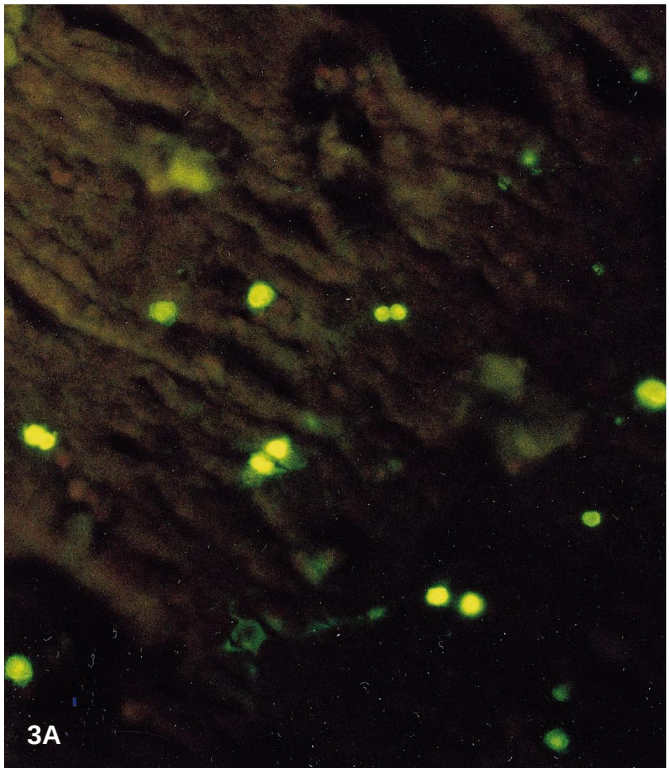


Fig. 1 Labelling of Ki-67 antigen in **A** well-differentiated and **B** poorly differentiated retinoblastoma cells. Ki-67 labelling disappears as the cells undergo necrosis, and well-differentiated cells also frequently express Ki-67 antigen. ×200



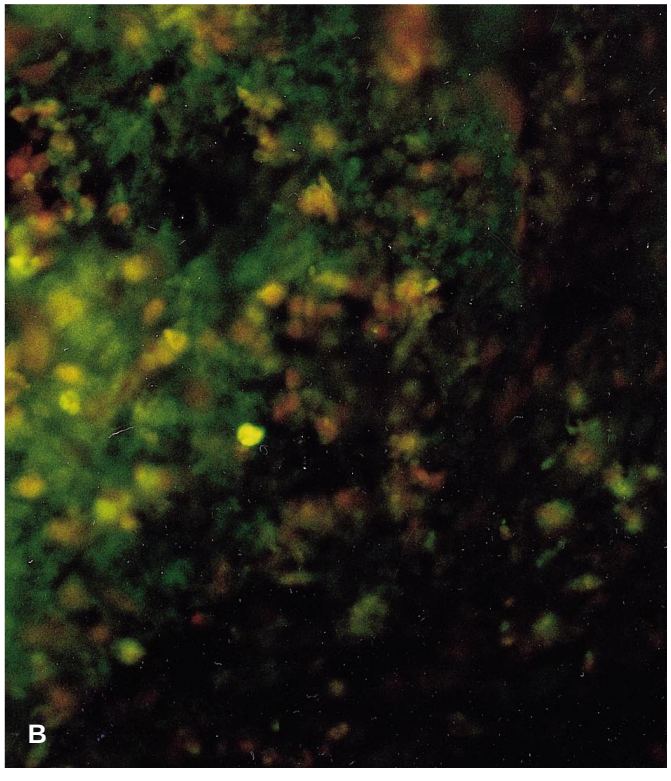


Fig. 3 Cases with **A** high and **B** low apoptotic indices, as defined by criteria of more or less than average of five apoptotic figures/microscopic field. Apoptotic cells show intense nuclear immunofluorescence after TUNEL staining using fluorescein-labelled anti-digoxigenin antibody. ×250

Results

The clinicopathological profiles of individual cases are summarized in Table 1. The fraction of the cells with positive nuclear staining for Ki-67 antigen was analysed, and PI ranged from 0.93% to 70.16% (mean 21.0±21.1%). The extent of Ki-67 antigen labelling was not related to degree of differentiation, and positive labelling was frequently observed even in cells comprising Flexner–Wintersteiner rosettes and those that were poorly differentiated (Fig. 1). Ki-67 antigen labelling was abruptly lost in cells undergoing necrosis. As shown in Table 2, there was a statistically significant difference in PI between group I (16.6±18.1%) and group II (32.7±25.1%). PI values in cases with and without *N-myc* amplification were 37.6±27.2% (*n*=6) and 17.3±18.1% (*n*=27), respectively; PI thus tended to be higher in *N-myc* amplified cases (*P*=0.03), although the number of cases is too small for significance. When cases were dichotomized according to PI, those with PI of more than 40% showed recurrence or died after an average of 23 months, and those with PI of less than 40% were disease free for an average of 76 months (Fig. 2). PI was also significantly associated with advanced tumour stage (Pearson's correlation coefficient = 0.61; $P<0.0001$).

Table 2 Comparison of clinicopathologic parameters between group I and group II

	Group I (*n*=24)	Group II (*n*=9)
Proliferative index*	16.6±18.1%	32.7±25.1
Apoptotic index (high/low)	10/14	3/6
N-myc amplification	5	1
Stage I	14 (58.4%)	2 (22.2%)
II	5 (20.8%)	2 (22.2%)
III	2 (8.3%)	1 (11.1%)
IV	3 (12.5%)	3 (33.4%)
V	0	1 (11.1%)

* $P<0.05$, between group I and group II, by *t*-test

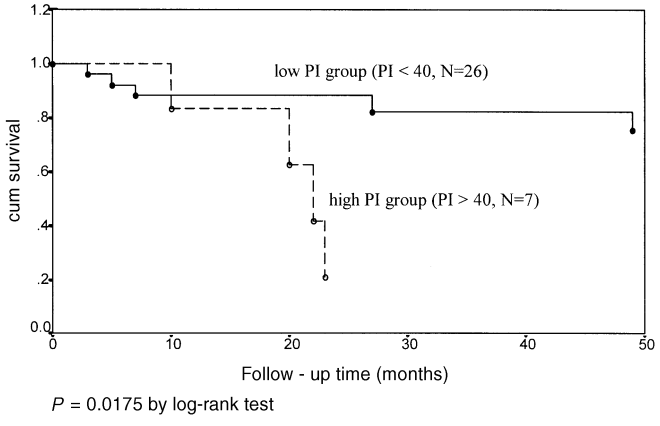


Fig. 2 Kaplan–Meier survival analysis of the patients according to proliferative index (*PI*). In cases with high PI (PI>40%), poor clinical outcome is clearly indicated

Apoptosis was clearly distinguished from background by intense fluorescence, and the number of cases with high and low AI were 13 (39.4%) and 20 (60.6%), respectively (Fig. 3). Five cases in group I (*n*=24) and one case in group II (*n*=9) had high AI, while in the remaining cases, AI was low. Although the majority of the cells undergoing characteristic geographic necrosis were labelled by TUNEL staining, demonstrating the occurrence of extensive apoptosis in the necrotic region, apoptosis per se was generally not prominent in solid, viable areas of a tumour. Cases with high and low AI had mean PI values of 21.4±25.1% and 20.8±18.8%, showing no relationship between AI and PI. *N-myc* amplification, tumour stage, and clinical outcome were not associated with AI.

Discussion

Although the involvement of anatomical structures including the choroid, sclera, ciliary body, iris, and anterior chamber influences prognosis [18, 22], there is no single histopathological prognostic variable that can be easily assessed during routine surgical diagnosis of retinoblastoma. Because loss of *rb* gene function is a major genetic factor in the development of retinoblastoma, and substantial data suggest that retinoblastoma protein ($p105^{rb}$) is a major coordinator of both cell proliferation and apoptosis [2, 15, 20], we postulated that both PI and AI might have prognostic significance. In the present study we evaluated the prognostic significance of PI and AI to ascertain their importance as prognostic markers in retinoblastoma. (These two parameters have frequently been reported to have prognostic significance in various human tumours.) We found that higher PI was associated with advanced tumour stage and poor clinical outcome, while AI was not a significant predictor of outcome.

In a given tumour, there are several markers that reflect the proliferative fraction: these include ^{3}H-thymidine uptake and bromodeoxyuridine labelling. Because they are easily applicable to paraffin-embedded sections, however, proliferating cell nuclear antigen (PCNA) and the Ki-67 antigen labelling index have been widely used in various tumours in the search for a prognostic indicator [12, 13]. Because a PI of over 40% is associated with poor clinical outcome, the evaluation of PI by the Ki-67 antigen labelling index will be helpful in identifying cases of retinoblastoma with a poor prognosis.

A poorer outcome in cases with higher PI was also observed in neuroblastoma, another major childhood tumour of neuroectodermal origin and one in which there is frequently amplification of *N-myc* in advanced stage tumours. The higher labelling index of proliferating cell nuclear antigen has been associated with *N-myc* amplification and poorer survival in neuroblastoma [17], and *N-myc* amplified primary neuroblastomas are characterized by a higher mitosis-karyorrhexis index [21]. It is interesting that *N-myc* amplification has a high prognostic significance in neuroblastoma, while it is of uncertain

value in retinoblastoma [1, 10, 24]. The tendency for association of *N-myc* amplification with higher PI suggests a possible relationship between *N-myc* and retinoblastoma cell proliferation, though the number of *N-myc*-amplified cases is limited in our study.

Apoptosis might not be so significant in determining the clinical behaviour of retinoblastoma, although it has been reported that increased apoptotic features defined a group of neuroblastomas with favourable prognosis [7]. *rb* Knockout mice die during embryonic development, and tissues which normally express high levels of *rb*, including those of the central and peripheral nervous system, show inappropriate cell proliferation and massive apoptosis [8, 9]. However, other molecules, such as *p53* and *bcl-2*, which play a crucial role in regulating apoptosis, are also expressed, and are believed to play some part in the development of retinoblastomas. Therefore, explanation of the oncological significance of *rb* as an inhibitor of apoptosis in retinoblastomas – whether chiefly by preventing entry of the S-phase or by direct suppression of apoptosis – becomes fairly complicated [16, 19].

Extensive ischaemic cell death is found in virtually all retinoblastomas and is subject to regional variation. Thus, the extent and significance of apoptosis must be interpreted with caution. Many of the cells affected were labelled by TUNEL, so that semiquantitative analysis was impossible; the evaluation of AI was therefore confined to the solid, viable area of an individual tumour. It is thus possible that the selection of an area of interest might have been a source of significant bias, but we believe that rather than being a genuine property of the tumour cells themselves, this type of extensive cell death is largely dependent on the vascular supply. Because the background was not amenable to evaluation, fluorescein labelling of AI did not permit quantitative ascertainment, but under a fluorescent microscope it was easy to distinguish between cases with high and low AI.

PI measured by immunohistochemical staining for Ki-67 antigen is a simple and valuable prognostic marker in retinoblastomas, and the relationship between *N-myc* amplification and PI should be further investigated in a large series of cases.

References

1. Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM (1984) Amplification of *N-myc* in untreated human neuroblastoma correlates with advanced disease stage. Science 224: 1121–1124
2. Chellappan SP, Hiebert S, Mudryi M, Horowitz JM, Nevins JR (1991) The E2F transcription factor is a cellular target for the RB protein. Cell 65:1053–1061
3. Fotedar R, Diederich L, Fotedar A, Ebel J-P (1996) Apoptosis and the cell cycle. Prog Cell Cycle Res 2:147–163
4. Friend SH, Bernards R, Rogelj S, Weinberg RA, Rapaport JH, Albert DM, Dryja TP (1986) A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature 323:643–646
5. Fung YK, Murphree AL, T'Ang A, Qian J, Hinrichs SH, Benedict WF (1987) Structural evidence for the authenticity of the human retinoblastoma gene. Science 236:1657–1661
6. Gavrieli Y, Sherman Y, Ben-Sasson SA (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119:493–501
7. Gestblom C, Hoehner JC, Påhlman S (1995) Proliferation and apoptosis in neuroblastoma: subdividing the mitosis-karyorrhexis index. Eur J Cancer [A] 31:458–463
8. Jacks T, Fazeli A, Schmitt EM, Bronson RT, Goodell MA, Weinberg RA (1992) Effects of an rb mutation in the mouse. Nature 359:295–300
9. Lee EY, Chang CY, Hu N, Wang YC, Lai CC, Herrup K, Lee WH, Bradley A (1992) Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. Nature 359:288–294
10. Lee WH, Murphree AL, Benedict WF (1984) Expression and amplification of the *N-myc* gene in primary retinoblastoma. Nature 309:458–460
11. Lee WH, Bookstein R, Hong F, Young LJ, Shew JY, Lee EY (1987) Human retinoblastoma susceptibility gene: cloning, identification, and sequence. Science 235:1394–1399
12. Lloveras B, Edgerton S, Thor AD (1991) Evaluation of in vitro bromodeoxyuridine labeling of breast carcinomas with the use of a commercial kit. Am J Clin Pathol 95:41–47
13. McCormick D, Chong H, Hobbs C, Datta C, Hall PA (1993) Detection of the Ki-67 antigen in fixed and wax-embedded sections with the monoclonal antibody MIB1. Histopathology 22:355–360
14. Mulder AH, Van Hootegem JS, Sylvester R, ten Kate FJW, Kurth KH, Ooms EC, Van der Kwast TH (1992) Prognostic factors in bladder carcinoma: histologic parameters and expression of a cell cycle-related nuclear antigen (Ki-67). J Pathol 166:37–43
15. Nevins JR (1992) E2F: a link between the Rb tumour suppressor protein and viral oncoproteins. Science 258:424–429
16. Nork TM, Poulsen GL, Millecchia LL, Jantz RG, Nicekells RW (1997) *p53* regulates apoptosis in human retinoblastoma. Arch Opthalmol 115:213–219
17. Oue T, Fukuzawa M, Kamata S, Okada A (1995) Immunohistochemical analysis of proliferating cell nuclear antigen expression in human neuroblastoma. J Pediatr Surg 30:528–532
18. Redler LD, Ellsworth RM (1973) Prognostic importance of choroidal invasion in retinoblastoma. Arch Ophthalmol 90: 294–296
19. Schlamp CL, Poulsen GL, Nork TM, Nickells RW (1994) Nuclear exclusion of wild-type p53 in immortalized human retinoblastoma cells. J Natl Cancer Inst 89:1530–1536
20. Shan B, Lee WH (1994) Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. Mol Cell Biol 14:8166–8173
21. Shimada H, Stram DO, Chatten J, Joshi VV, Hachitanda Y, Brodeur GM, Lukens JN, Matthay KK, Seeger RC (1995) Identification of subsets of neuroblastomas by combined histopathologic and *N-myc* analysis. J Natl Cancer Inst 87:1470–1476
22. Stannard C, Lipper S, Sealy R, Sevel D (1979) Retinoblastoma: correlation of invasion of the optic nerve and choroid with prognosis and metastases. Br J Ophthalmol 63:560–570
23. Staunton MJ, Gaffney EF (1995) Tumor type is a determinant of susceptibility to apoptosis. Am J Clin Pathol 103:300–307
24. Yokoyama T, Tsukahara T, Nakagawa C, Kikuchi T, Minoda K, Shimatake H (1989) The *N-myc* gene product in primary retinoblastomas. Cancer 63:2134–2138
25. Zimmerman LE (1985) Retinoblastoma and retinocytoma. In: Spencer WH (ed) Ophthalmic pathology: an atlas and textbook. Saunders, Philadelphia, pp 1292–1351