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Quantitative Analysis of Proliferation, Apoptosis, and Angiogenesis in Retinoblastoma and Their Association with the Clinicopathologic Parameters

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Purpose: Quantitative analyses of proliferation, apoptosis, and angiogenesis, which may be important for the prognosis of retinoblastoma, were performed and possible associations with some well-known clinicopathologic parameters were investigated.

Methods: Fifty-three pathology specimens (43 enucleations, 10 exenterations) were evaluated by immunohistochemical methods. The proliferative index was detected by Ki67 antibody staining. The apoptotic index was calculated by the in situ terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) method, and angiogenesis was detected by CD34 antibody staining.

Results: The mean proliferative index was 37.63 ± 11.12, the mean apoptotic index was 2.67 ± 1.18, and the microvessel density and mean vascular area were determined as 3.14 ± 1.4 and 38.73 ± 12.70, respectively. Statistical analysis showed that the proliferative index was directly proportional to the tumor dimensions ($P = .001$). In addition, the tumor dimensions were larger in cases where the apoptotic index was below 2.4% ($P = .011$). In cases where the apoptotic index was over 2.4%, no metastasis was observed and also a lower proliferative index was found ($P = .014$).

Conclusions: Proliferation appears to be more important than apoptosis and angiogenesis in determining the tumor dimensions. The apoptotic index may be an important predictor of metastasis, and may be useful in the follow-up of bilateral cases with 1 eye enucleated. **Jpn J Ophthalmol 2003;47:565–571**

Key Words: Angiogenesis, apoptosis, in situ terminal deoxynucleotidyl transferase mediated dUTP nick end labeling, proliferative index, retinoblastoma.

Introduction

Any genetic event acting on the two alleles of the retinoblastoma gene (RB1) and leading to the functional loss of its product, the retinoblastoma protein (pRb), ultimately results in the formation of retinoblastoma.[1] The nuclear phosphoprotein pRb not only suppresses proliferation through transcription factors, but also modulates apoptosis and cellular differentiation.[2,3]

The key aspect of malignancy is uncontrolled tumor growth. Tumor growth is a result of the imbalance between cell proliferation and cell death, either because of suppressed cell death or increased cellular proliferation. These two variables of the equilibrium play important roles as prognostic factors in many human malignancies.[4,5]

The proliferation rate is one of the most important indicators of the biologic behavior of malignancies. Although it can be observed under a light microscope, low reproducibility and high interobserver differences necessitated new and accurate indicators of the proliferation rate. Ki67 is a nuclear protein that is expressed at the beginning of the G_1 phase, increases through the cell cycle, and disappears just after mitosis in a short period of time.[6,7] Thus, proliferating cell fraction can be determined immunohistochemically by using antibodies against Ki67 protein.[7]

Apoptosis and necrosis are the two major mechanisms of cell loss in any neoplastic tissue.[8] Apoptosis is a precisely regulated and energy-dependent type of programmed cell death, and has characteristic morphological criteria, including condensation of the nuclear chromatin and cytoplasm and the formation of protuberances on the surface of individual cells. These membrane-bound, well-preserved cell remnants (apoptotic

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bodies) are subsequently taken up by neighboring cells.[9] Internucleosomal DNA breaks, producing fragments that are multiples of 180 bp, are the major biochemical features of apoptosis and make it possible to identify apoptotic cells by immunohistochemical methods or DNA gel electrophoresis.[10]

Angiogenesis is another prognostic parameter. In solid tumors, tumoral angiogenesis is required for tumor growth and metastasis.[11] Angiogenesis is a complex, multi-step process involving extracellular matrix remodelling, endothelial cell migration and proliferation, capillary differentiation, and anastomosis.[12] CD34, a well-known myeloid progenitor cell antigen, can be detected immunohistochemically on the artery, vein, and capillary walls for assessment of microvessel density (MVD), for quantitative analysis of angiogenesis.[13]

This immunohistochemical study presents a quantitative analysis of proliferation, apoptosis, and angiogenesis, and their relationship with tumor diameters, differentiation type, necrosis rate, calcification, choroidal, scleral, and optic nerve invasion, presence of metastasis, and surgery type.

Materials and Methods

A total of 53 enucleation and exenteration specimens obtained from patients with retinoblastoma treated between 1995 and 2001 at the Ocular Oncology Service of the Ophthalmology Department of Hacettepe University Hospital, formed the study group. Clinical data were obtained from the medical records, including age, sex, laterality, presence of metastasis, and operation type.

For histopathological and immunohistochemical evaluation, serial 5-μm–thick sections were obtained from representative paraffin blocks. Formalin-fixed and paraffin-embedded sections from all specimens were stained by hematoxylin eosin. The sections were evaluated under a light microscope and neoplasms were grouped as differentiated and undifferentiated. Differentiated tumors were characterised by the presence of Flexner-Wintersteiner rosettes and fleurettes, and undifferentiated tumors were composed of small cells with hyperchromatic nuclei and scanty cytoplasms. The extent of necrosis and calcification in tumor tissue was noted and the area of the tissue section occupied by necrosis was recorded as a percentage (%). The presence of invasion of choroid, sclera, and optic nerve surgical borders was noted.

For immunohistochemical evaluation of Ki67 and CD34, two series of sections were deparaffinized and rehydrated through xylene and graded alcohol. Endogenous peroxidase was quenched with 3.0% hydrogen peroxidase in methanol for 10 minutes. Following phosphate-buffered saline (PBS) rinses, sections were immersed in citrate buffer (10 mM citric acid monohydrate, adjusted to pH 6.0) and heated in a microwave at boiling point for 20 minutes. Then the microwave-irradiated sections were cooled to room temperature and washed with PBS. They were then incubated in protein blockage solution (Zymed Laboratories, San Francisco, CA, USA) for 10 minutes. Sections were incubated with primary antibodies; mouse anti-human monoclonal antibody (Neomarkers, Fremont, CA, USA) diluted 1:100 in PBS for Ki67 immunostaining and mouse anti-human monoclonal antibody (DAKO, Glostrup, Denmark) diluted 1:25 in PBS for CD34 immunostaining. No primary antibody was added on sections used as negative controls. After PBS rinse, sections were treated with biotinylated second antibody (Zymed) for 10 minutes and enzyme conjugate (HRP-streptavidin, Zymed) for 10 minutes at room temperature. The reaction was developed with diaminobenzidine (DAKO, Carpinteria, CA, USA) and slides were counterstained in hematoxylin for 5 seconds.

For evaluation of the proliferative index (PI) in each section, a minimum of 2000 cells were counted at ×40 magnification on the monitor using a video system. The fraction (%) of the cells that showed positive nuclear staining for Ki67 antigen was considered to be the PI (Figure 1A).

For assessment of microvessel density (MVD) and mean vascular area (MVA), the entire area of the immunostained tumor at ×10 magnification was scanned and the five separate, most highly vascularized areas (hot spots) were identified. Vessels were counted at ×40 magnification on the monitor (Sony PVM-1371QM) using a video system (Sony DXC-101P). Any immunolabeled vessel, clearly separate from an adjacent one either totally inside the monitor or touching the borders, was counted as a microvessel. The mean of five counts was used for the analysis of MVD. For analysis of MVA the mean of the endothelial cell count of these five areas was used (Figures 1C and 1D).

For evaluation of apoptosis, a commercially available kit (ApopTag-In situ Apoptosis Detection Kit/Peroxidase; Oncor, Gaithersburg, MD, USA) was used. The technique was performed as described elsewhere.[10] Briefly, formalin-fixed and paraffin-embedded 5-μm tissue sections were deparaffinized. Nuclear proteins were stripped from DNA by incubating in proteinase K for 30 minutes, and endogenous peroxidase was blocked with 2% H_2O_2. Sections were incubated in a buffer containing terminal deoxynucleotidyl transferase (TdT) and digoxigenin-labeled dUTP, followed by anti-digoxigenin conjugated peroxidase treatment. Diaminobenzidine was used as chromogen, and background staining was done with methyl green. For negative controls TdT reaction solution was replaced by distilled water.

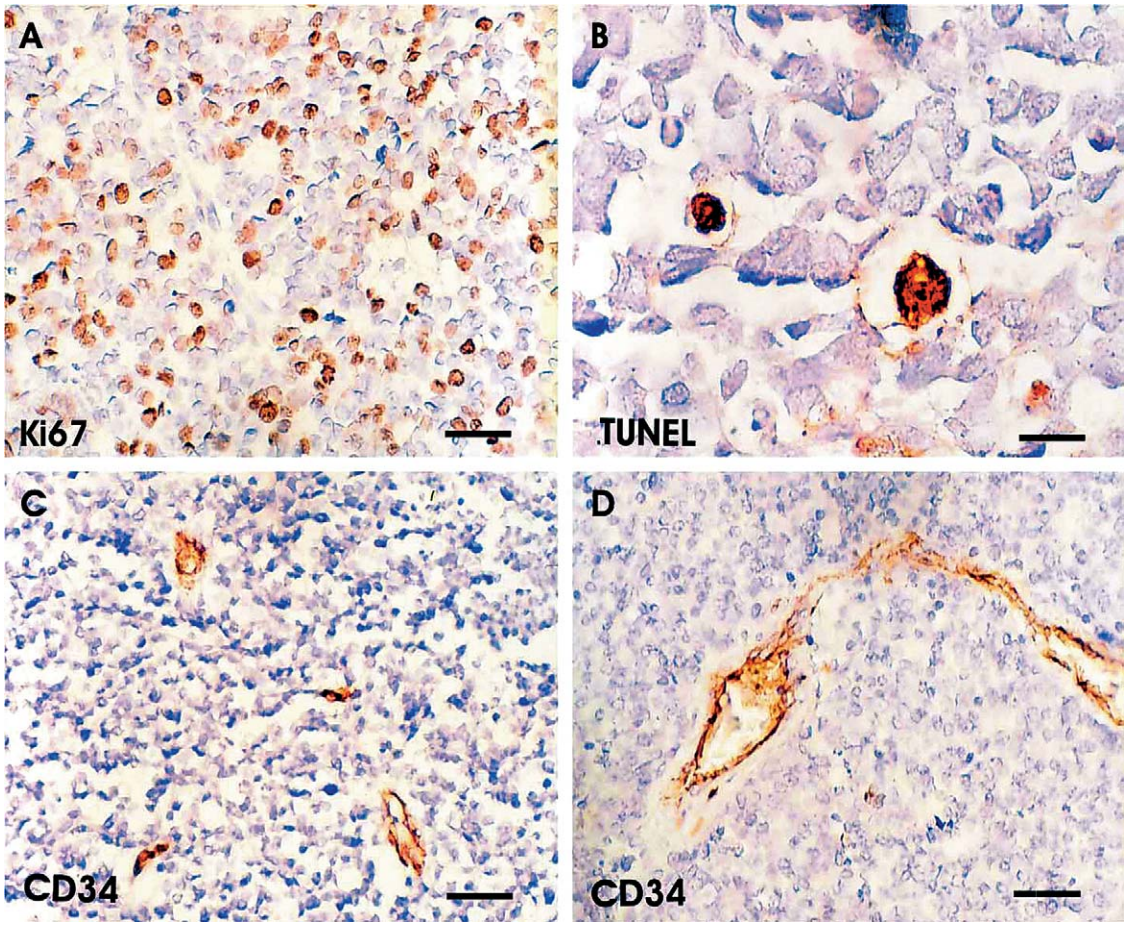

Figure 1. Photomicrographs illustrating the immunohistochemical staining of Ki67 expression (**A**), in situ terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) staining (**B**), and CD34 expression (**C**,**D**). (**A**) Positive nuclear Ki67 staining of tumor cells. Bar = 25 µm. (**B**) Two apoptotic neoplastic cells showing TUNEL-positive staining. Bar = 10 µm. (**C**,**D**) Vascular endothelial staining with CD34. Bars = 25 µm).

In non-necrotic viable tumor tissue, at least 1000 cells were counted from the same slide for each specimen using an ×100 objective. Apoptotic cells were defined by the presence of perinuclear chromatin condensation and apoptotic bodies, while cells showing more diffuse cytoplasmic staining were considered as necrotic and were not counted (Figure 1B). The fraction (%) of the apoptotic cells was considered to be the apoptotic index (AI).

SPSS for Windows version 10.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. The relationship between clinicopathologic and immunohistochemical parameters was analysed by χ^2 test, Fisher's Exact Test, independent samples *t*-test, and Mann–Whitney *U*-test. $P < .05$ was considered as statistically significant.

Results

Clinical characteristics of patients and histopathological features of retinoblastoma specimens are presented in Table 1. The relationships between clinical, histopathological, and immunohistochemical data were statistically analyzed (Table 2). According to the data reviewed, undifferentiated tumors predominated in exenteration specimens ($P = .034$) and in those tumors with basal diameters >15 mm ($P = .001$).

A necrosis rate >50% was detected in cases showing choroidal invasion ($P = .047$) and in cases showing metastasis ($P = .044$). Tumor diameters >15 mm were more frequently associated with choroidal invasion ($P = .036$), had a higher necrosis rate ($P = .002$) and calcification ($P = .001$). Metastasis was more frequent in undifferentiated cases and in cases whose tumor diameters were >15 mm, but the difference was not statistically significant.

The mean (± SD) proliferative index of 41 cases was found to be 37.63 ± 11.12 (range, 10.25–58.7%). The proliferative index was directly proportional to tumor dimensions and was higher in tumors with diameters >15

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Table 1. Clinical Characteristics of Retinoblastoma Patients and Histopathological Features of Specimens Analyzed in the Study

Characteristics	No. of Cases (%)
Sex	
Male	26 (49)
Female	27 (51)
Age	
Mean (range)	2.4 years (1–8 years)
Laterality	
Bilateral	23 (43.3)
Unilateral	30 (56.7)
Metastasis	
Orbital	9 (17)
Central nervous system	1 (1.9)
Bone marrow	2 (3.8)
Invasion	
Choroidal	12 (22.6)
Scleral	7 (13.2)
Optic nerve resection border	8 (15.1)
Operation type	
Enucleation	43 (81.8)
Exenteration	10 (18.2)
Differentiation	
Differentiated	28 (52.8)
Undifferentiated	25 (47.2)
Tumor diameter	
>15 mm	35 (66)
<15 mm	18 (34)
Necrosis	
>50%	14 (26.5)
<50%	39 (73.5)
Calcification	
+	37 (69.8)
−	16 (30.2)

Table 2. Statistical Associations Between Clinicopathologic and Immunohistochemical parameters

Relationship Examined	No. of Specimens	Statistical Association
Undifferentiated RB and tumor diameter (>15 mm)	53	$P = .001$*
Undifferentiated RB and operation type (exenteration)	53	$P = .034$*
Undifferentiated RB and metastasis	53	$P = .067$*
Undifferentiated RB and choroidal invasion	53	$P = .055$*
Necrosis rate (>50%) and choroidal invasion	53	$P = .047$*
Necrosis rate (>50%) and metastasis	53	$P = .044$*
Tumor diameter (>15 mm) and choroidal invasion	53	$P = .036$*
Tumor diameter (>15 mm) and necrosis rate	53	$P = .002$*
Tumor diameter (>15 mm) and calcification	53	$P = .001$*
Higher PI and tumor diameter	41	$P = .001$†, $t = 5.980$
PI (>35%) and AI	32	$P = .014$‡, $U = 28.0$
Lower A1 and tumor diameter (>15 mm)	36	$P = .195$†, $t = 1.323$
Lower A1 and operation type (exenteration)	36	$P = .072$‡, $U = 56.0$
Lower A1 and metastasis	36	$P = .005$‡, $U = 27.0$
A1 (<2.4%) and metastasis	36	$P = .024$*
A1 (<2.4%) and tumor diameter (>15 mm)	36	$P = .011$*
AI (<2.4%) and PI	32	$P = .014$†, $t = 2.601$
Lower MVD and tumor diameter (>15 mm)	48	$P = .043$†, $t = 2.079$
Higher MVA and undifferentiated RB	48	$P = .039$†, $t = 2.121$

RB: retinoblastoma, PI: proliferative index, AI: apoptotic index, MVD: microvessel density, MVA: mean vascular area.
*Fisher's Exact Test.
†Independent samples *t* test.
‡Mann-Whitney *U*-test.

mm ($P = .001$). The apoptotic index was determined to be lower in cases whose PI was over 35% ($P = .014$).

The mean (± SD) apoptotic index was calculated as 2.67 ± 1.18 (range, 1.3–7.8%). The apoptotic index was definitely lower in cases showing metastasis ($P = .005$). AI was also lower in tumors with basal diameters >15 mm and in exenteration specimens, but the difference was not statistically significant.

In cases whose AI was under 2.4%, metastasis was more frequent ($P = .024$) and tumor diameters were larger ($P = .011$). There was no metastasis in cases whose AI was >2.4%. PI was also higher in cases whose AI was <2.4% ($P = .014$).

Microvessel density and mean vascular area of 48 cases were determined as 3.14 ± 1.4 (range, 1–11) and 38.73 ± 12.70 (range, 8.80–66.40), respectively. MVD was found to be lower in tumors with diameters >15 mm ($P = .043$) and MVA was found to be higher in undifferentiated tumors ($P = .039$).

Discussion

Histopathological findings including involvement of the optic nerve, choroid, sclera, ciliary body, iris, and anterior chamber were used to determine the biological behavior and prognosis of retinoblastoma for many years.[14,15]

In the absence of pRb, the cell cycle cannot be stopped at the G_1 restriction point and cells go through the S phase and proliferate continuously. Ki67 immunostaining has the advantage of detecting precisely the proliferating cell population during the cell cycle, except for the G_0 phase.[6]

The proliferative index and the apoptotic index may have prognostic significance along with other clinicopathologic parameters. Although the proliferative index has been correlated with the clinical course in many human malignancies,[16–21] there are only a few quantitative studies performed on retinoblastoma. Kim et al[22] investigated the prognostic importance of PI and AI in retinoblastoma patients. PI was found to be 21% for the whole group, ranging from 0.93% to 70.16%. No correlation between differentiation type and PI could be established, but PI was found to be higher in patients with recurrence. Disease-free survival of patients whose PI was >40% was found to be shorter. PI was also directly correlated with the tumor grade, although AI did not correlate with either the tumor grade or the clinical results.[22] In a recent study, Orjuela et al[23] reported a highly significant correlation between advanced clinical stage and a higher Ki67 proliferative index. Among children with unilateral disease, the mean PI was higher in children with advanced clinical disease (stage 3–4) than in those with earlier stage disease (stage 1–2). However, among children with bilateral disease, the mean PI was not significantly higher for children at an advanced clinical stage. It has been suggested that unregulated cell growth contributes to a more aggressive disease phenotype of tumors without a germinal loss of pRb.[23]

In our study, PI was higher in patients with tumor diameters >15 mm. Although AI was lower in the same group of patients, this result was not statistically significant. However, there was an inversely proportional correlation between AI and PI >35%. These results suggest that proliferation but not apoptosis may be the determining factor in tumor growth. Apoptosis might have balanced proliferation in tumors with a PI <35%; however, the mechanism triggering proliferation may also suppress apoptosis in cases with PI >35%. Necrosis rate was also higher in tumors with a largest diameter >15 mm, and MVD was lower in tumors with a diameter >15 mm. These results can also explain why the main cellular death mechanism was necrosis in larger tumors because of vascular insufficiency and ensuing hypoxia. Also, tumors with a basal diameter >15 mm had a higher rate of choroidal invasion, and PI was higher in tumors with choroidal invasion, but that was not statistically significant.

Although apoptosis is a common feature of all malignant tumors, only a few quantitative studies have been performed regarding retinoblastoma. Based on light microscopic findings, Madigan and Penfold[24] reported an AI of 4.8 ± 1.9 (range, 2.5–7.2) in a series of 5 cases with retinoblastoma. In another study, the rate of apoptosis was classified qualitatively as high (5 or more apoptotic counts per field under ×250 magnification) or low (fewer than 5 per field) rather than using the quantitative data by Kim et al.[22] The other study involved 13 retinoblastoma specimens, and apoptotic cell death was shown by the TUNEL method. It was reported that differentiated tumors had a low (<1%), and undifferentiated cases had a higher, (>8%) rate of apoptosis.[25]

Necrosis in tumor tissue mainly results from ischemia, and mild hypoxia is a known factor inducing apoptosis.[5] When we grouped the tumors having necrosis under and over 50%, AI was higher in the group with a necrosis rate under 50%, but the difference was not statistically significant.

The apoptotic index was definitely lower in cases with metastasis. No metastasis was observed in patients whose AI was >2.4%. Although AI was low in these cases, the necrosis rate was higher and this relation may indicate rapid tumor growth because of suppressed programmed cell death. Necrosis might have been induced by insufficiency of the vascular supply.

Two of the three cases who died during follow-up and all of the cases who had metastasis had an AI <2.4%. Therefore, an AI <2.4% can be an indicator of poor prognosis, and especially for bilateral cases it can be an indicator for close follow-up for metastasis. In exenteration specimens, AI was lower than in enucleation specimens and this finding can indicate an aggressive biological behavior. There was no correlation between metastasis and PI. Aggressive behavior of the tumor may be induced by the diminished effect of programmed cell death. Although metastasis was more prevalent among undifferentiated tumors, AI and PI exhibited no significant difference between differentiated and undifferentiated tumors.

Tumor growth, progression, and metastasis can be possible only with adequate vascular supply.[11] Many investigators observed the same association of increasing tumor vascularity with various measures of tumor aggressiveness, such as higher stage at presentation, greater incidence of metastasis, and decreased patient survival.[26] An association with MVD, quantified by microvessel counting, and tumor prognosis or metastasis has been reported in several human tumors.[27–33]

In one study, aqueous humor from 30 enucleated eyes of retinoblastoma patients and 9 cell cultures from retinoblastoma patients were tested for angiogenesis activity using capillary endothelial cell migration assays. Aqueous humor from 90% of the retinoblastoma eyes stimulated endothelial cell migration, compared with only 25% of control eyes.[34] Several other studies have shown that certain intraocular tumors, such as retinoblastoma, display angiogenesis capacity before clinically evident neovascular changes.[35]

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In our study, MVD was higher in tumors with diameters <15 mm. Although it was expected that larger tumors need more vascular supply and would have higher MVD, it is possible that the rate of proliferation exceeds the rate of angiogenesis in these tumors. This theory is also supported by the higher rate of necrosis in larger tumors, which is induced by hypoxia.

Mean vascular area was higher in undifferentiated retinoblastomas. When we consider that choroidal invasion and metastasis were seen more frequently in undifferentiated cases, higher mean vascular area may support the fact that cases with undifferentiated retinoblastoma have a well-developed vascular supply.

Conclusion

This study provides quantitative data about the rate of proliferation, apoptosis, and angiogenesis, and their association with the well-known clinicopathologic parameters concerning retinoblastoma. It revealed that tumor dimensions were larger and choroidal invasion was more frequent in undifferentiated cases. Proliferation is more important than apoptosis and angiogenesis in determining the tumor growth. For bilateral cases, the apoptotic index may be an important indicator of metastasis after enucleation of 1 eye.

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