Virchows Arch (1995) 426:571–575

ORIGINAL ARTICLE

Kenshi Yuge · Motomaro Nakajima
Yoshiko Uemura · Hirohiko Miki · Masanobu Uyama
Airo Tsubura

Immunohistochemical features of the human retina and retinoblastoma

Received: 17 January 1995 / Accepted: 18 April 1995

Abstract The immunohistochemical features of 24 retinoblastoma specimens from 22 patients, 15 with unilateral and 7 with bilateral disease, were examined by the labelled streptavidin biotin (LSAB) method and compared with those of specimens from the remaining morphologically normal retina. In the normal retina, S-100 protein, glial fibrillary acidic protein (GFAP) and vimentin were detected in astrocytes and/or Müller cells. Neurofilament protein was seen in axons of the ganglion cells, synaptophysin was present in both plexiform layers, bcl-2 oncoprotein was seen in ganglion cells and bipolar cells, and neuron-specific enolase (NSE) was detected in ganglion cells, bipolar cells and photoreceptor cells and in their cell processes. While retinoblastoma (Rb) protein expression was noted in ganglion cells, bipolar cells, and some photoreceptor cells, p53 protein was not expressed at all. In all retinoblastomas, strong NSE expression and weak bcl-2 expression was observed in almost all tumour cells and synaptophysin was localized in rosette-forming cells, while tumour cells were devoid of S-100, GFAP, vimentin and neurofilament protein. These findings support the view that retinoblastomas are composed of neuron-committed cells. In addition, no Rb protein expression was detected in retinoblastomas, whereas p53 expression was found in 18 cases (75%).

Key words Retinoblastoma · Immunohistochemistry · Rb protein · p53

Introduction

Retinoblastoma is the most common ocular malignancy in newborn babies and infants. It arises during retinal development, and the primitive phenotype is consistent with the appearance of the cells in the 6- to 10-week fetal retina [17]. More than a century ago, Virchow (1864) suggested that retinoblastomas originated in the supporting glial cells of the retina and designated the tumour a "glioma of the retina" [25]. Recently, culture studies have given rise to the opinion that retinoblastoma might originate from multipotential precursor cells of retinal neurons and glia [7, 13, 21]. Retinoblastoma in situ however, has neuronal characteristics but does not generally exhibit glial properties as determined by immunohistochemistry [6, 8–12, 14], and glial cells found in the tumour are considered to be reactive but not neoplastic. The histogenesis of retinoblastoma is thus still in dispute.

Retinoblastoma was the first cancer to be identified with a tumour suppressor gene, *Rb1*, and its protein product, $p110^{Rbl}$ has been isolated [1]. Retinoblastoma arises when both alleles of the suppressor gene are inactivated within a single cell. In contrast, increased levels of p53 protein are found in other human malignant tumours [18]. However, the expression of Rb protein and p53 protein in retinoblastoma in situ has not been examined.

To reinvestigate the cell phenotype and to clarify oncoprotein expression, we examined 24 human retinoblastomas in situ using a panel of immunohistochemical markers with well-defined specificities, including S-100 protein, glial fibrillary acidic protein (GFAP), and vimentin as a glial marker, synaptophysin, bcl-2 and NSE as a neuronal marker; we also examined them for Rb protein and p53 protein expression.

Materials and methods

Between 1972 and 1994, a total of 24 retinoblastoma specimens from 22 patients were collected from the surgical pathological files of the Surgical Pathology Division, Kansai Medical University Hospital. In 15 patients retinoblastoma was unilateral and in 7, bilateral. Among the 7 bilaterally affected patients, specimens from both eyes were available in 2 cases but from only one eye in the remaining 5. All the specimens were enucleated globes. The

K. Yuge · M. Nakajima · H. Miki · M. Uyama
Department of Ophthalmology, Kansai Medical University, Moriguchi, Osaka 570, Japan

Y. Uemura · A. Tsubura (✉)
Department of Pathology, Kansai Medical University, Moriguchi, Osaka 570, Japan

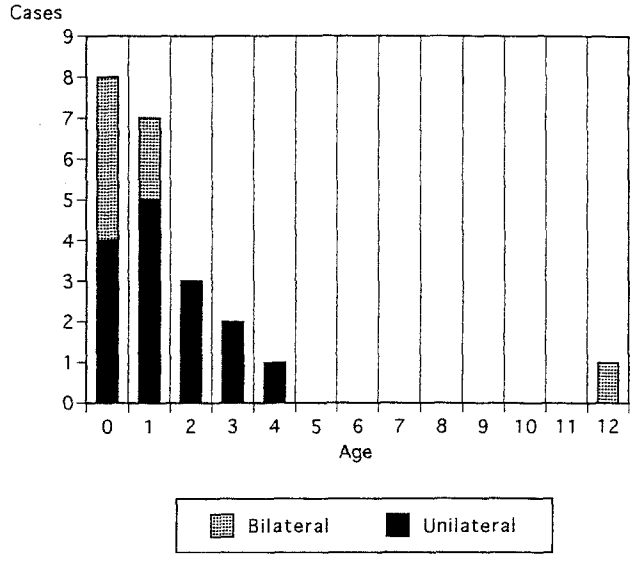


Fig. 1 Laterality and age distribution of 22 retinoblastoma patients studied

Table 1 Panel of monoclonal antibodies and polyclonal antisera used (*GFAP* glial fibrillary acidic protein, *NSE* neuron-specific enolase, *Rb* retinoblastoma)

Specificity	Clone	Source	Dilution
S-100[a]		Dako, Glostrup, Denmark	×200
GPAF	6F2	Dako, Glostrup, Denmark	×50
Vimentin	V9	Dako, Glostrup, Denmark	×250
Neurofilament	2F11	Dako, Glostrup, Denmark	×50
Synaptophysin	SY38	Boehringer Mannheim, Germany	×5
bcl-2	124	Dako, Glostrup, Denmark	×40
NSE[a]		Dako, Glostrup, Denmark	×100
Rb protein	84-B3-1	Novocastra, Newcastle upon Tyne, UK	×50
p53 protein	BP53-12	Bioprobe, Amstelveen, Netherlands	×300

[a] Polyclonal antiserum

patients were beween 0 and 12 years old (average 1.6±2.6), and the male-to-female ratio was 12:10. Figure 1 shows the laterality and age distribution. In general, bilateral cases were found in the younger patients, but one patient with bilateral tumour was 12 years old. All specimens were fixed in 10% formalin and were subjected to routine paraffin processing. Serially cut sections 4 μm thick were carefully heated (60 °C, 5 min) to assist adherence to silane-coated slides; they were then dewaxed, stained with haematoxylin and eosin, and used for the immunohistochemical study.

The mouse monoclonal antibodies and rabbit polyclonal antisera used are listed in Table 1. The polyclonal anti-S-100 antiserum purified from the cow brain cross-reacts strongly with human S-100 A and B. The monoclonal anti-glial fibrillary acidic protein (GFAP) antibody purified from human brain reacts with the 52-kDa intermediate filament protein GFAP and the monoclonal anti-vimentin antibody purified from porcine eye lens reacts with the 57-kDa intermediate filament protein of the human vimentin. The monoclonal anti-neurofilament antibody purified from human brain reacts specifically with the 200- and 70-kDa components of the three major subunits (200, 160 and 70 kDa) present in the neurofilaments. The monoclonal anti-synaptophysin antibody purified from bovine brain reacts with human presynaptic vesicles. Monoclonal anti-bcl-2 antibody was prepared from a synthetic peptide sequence comprising amino acids 41–54 of bcl-2 protein. Polyclonal anti-neuron-specific enolase (NSE) purified from human brain reacts with the gamma subunits of NSE. Monoclonal anti-retinoblastoma (*Rb*) gene protein was prepared from a synthetic peptide representing an antigenic site on the retinoblastoma gene molecule, and monoclonal anti-p53 antibody was produced from a human recombinant wild-type p53.

In immunocytochemistry, endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 in ethanol for 10 min after dewaxing, with subsequent washing in distilled water for 5 min. The sections were then incubated with the appropriately diluted primary antibody in a moist chamber at room temperature for 1 h. Subsequently, a labelled streptavidin biotin staining kit (Dako, Carpenteria, Calif.) was used according to the manufacturer's instructions. The reaction products were visualized with freshly prepared 0.02% 3.3'-diaminobenzidine tetrahydrochloride (DAB; Wako Pure Chemical, Osaka, Japan) containing 0.007% H_2O_2, and the sections were counterstained with Gill's haematoxylin. Antigen retrieval was necessary for demonstration of neurofilaments, bcl-2, Rb protein and p53. In brief, prior to incubation with each antibody, the sections were placed in plastic staining jars containing citrate buffer solution (pH 6.0). The jars were then covered with loose-fitting caps and heated in a microwave oven for two 10-min cycles with an interval of 1 min between cycles to check the fluid level. After being heated, the jars were allowed to cool for 15 min. In preliminary stainings, this procedure was found to enhance the intensity of the specific reaction, and findings were consistently reproducible when antigen retrieval in a microwave oven was adequate. For a negative control, serial sections were incubated with non-immune mouse or rabbit serum at a comparable dilution instead of the primary antibodies. In addition, the ubiquity of the microscopically normal-appearing retina present in retinoblastoma specimens served as a built-in positive control.

Results

Normal retina

In a total of 22 specimens, there was enough morphologically normal retina to allow study of its antigen expression. Table 2 summarizes the characteristics of the immunostaining. S-100 protein showed strong staining in astrocytes in the nerve fibre layer (Fig. 2a). Immunostaining for GFAP was similar to that for S-100, but in addition, vertically distributed Müller cells were also weakly positive (Fig. 2b). Vimentin positivity was seen in longitudinal fibres representing astrocytes in the nerve fibre layer, and in the radical fibres of Müller cells (Fig. 2c). No neuronal cells had positive results with S-100, GFAP and vimentin. A positive reaction for neurofilament protein was seen in fibres corresponding to the axons of ganglion cells in the nerve fibre layer (Fig. 2d). The stainability was intense near the optic nerve papilla. Synaptophysin showed reactivity in the two plexiform layers, while perikarya of neuronal cells remained negative (Fig. 2e). Weak expression of bcl-2 was seen in ganglion cells and bipolar cells (Fig. 2f). NSE was found strongly in perikaria of the ganglion cells, bipolar cells and photoreceptor cells and also in their cell processes (Fig. 2g). Astrocytes and Müller cells remained negative with neurofilament protein, synaptophysin, bcl-2 and NSE. Rb protein showed nuclear staining in ganglion cells and bipolar cells, while the photoreceptor cells dis-

Table 2 Immunohistochemical reaction patterns of the normal retina and retinoblastomas (++ strong staining, + weak staining, – negative)

	S-100	GFAP	Vimentin	Neurofilament	Synaptophysin	bcl-2	NSE	Rb	p53
Normal retina									
Müller cell	–	+	+	–	–	–	–	–	–
Astrocyte	++	++	+	–	–	–	–	–	–
Ganglion cell axon	–	–	–	++	–	–	++	–	–
Ganglion cell	–	–	–	–	–	+	++	++	–
Inner plexiform layer	–	–	–	–	++	–	++	–	–
Bipolar cell	–	–	–	–	–	+	++	++	–
External plexiform layer	–	–	–	–	++	–	++	–	–
Photoreceptor cell	–	–	–	–	–	–	++	+	–
Cone and rod	–	–	–	–	–	–	++	–	–
Retinoblastoma	–	–	–	–	+[a]	+[b]	+[c]	–	+/–[d]

[a] Positive on rosette-forming cells
[b] Weak on all tumour cells
[c] Strong on all tumour cells
[d] Positive in 18 cases (75%)

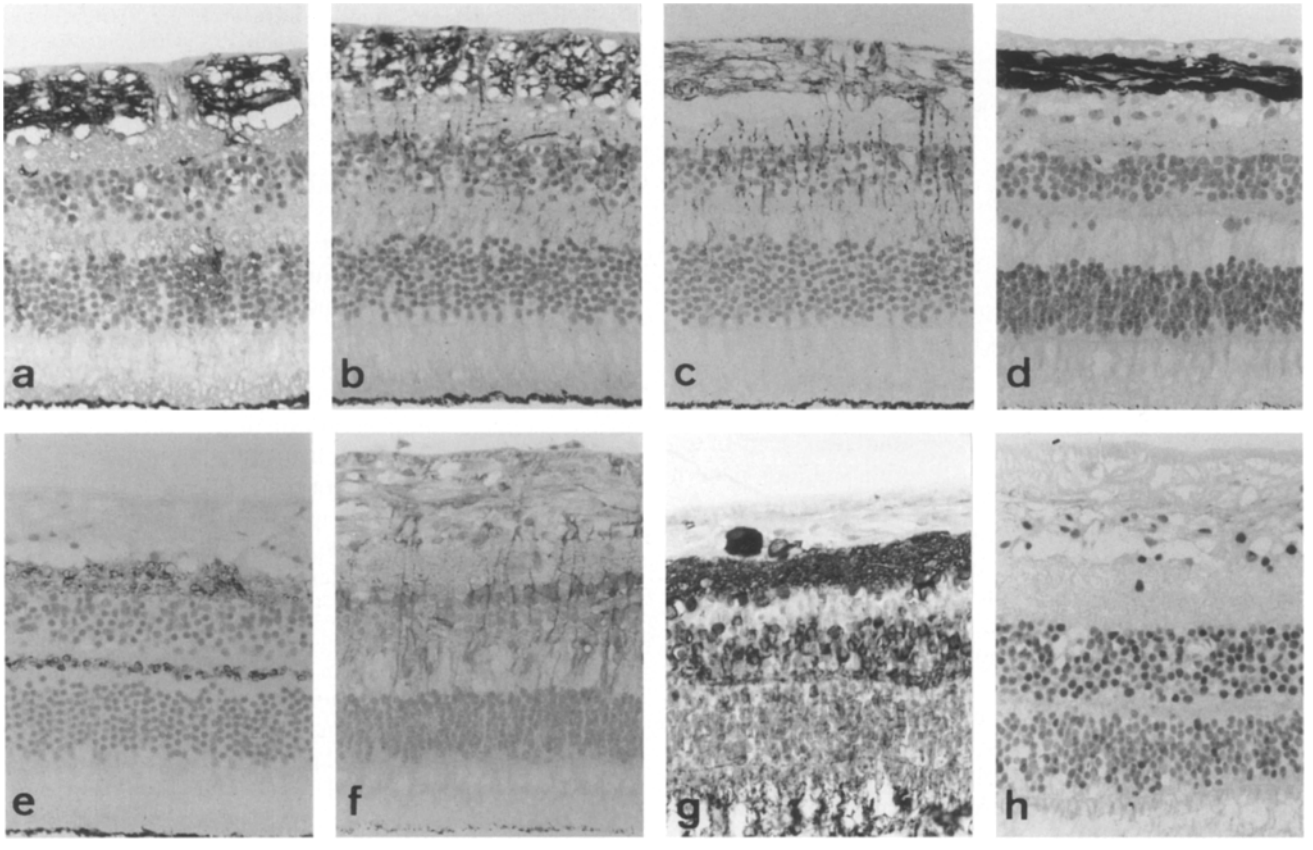
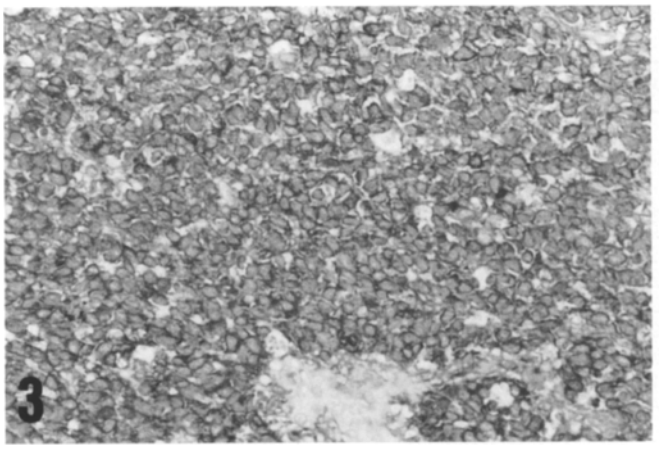


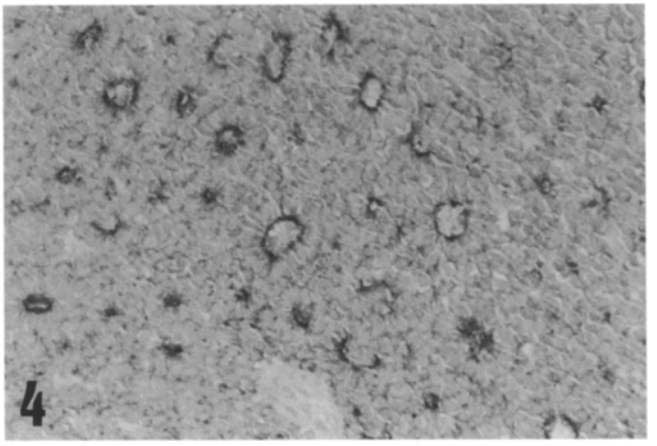
Fig. 2a–h Normal retina. ×200. **a** Astrocytes in the nerve fibre layer are strongly stained (S-100). **b** In addition to astrocytes, Müller cells are also stained, but weakly. (GFAP). **c** Astrocytes and Müller cells are positively stained (vimentin). **d** Axons of the ganglion cells are strongly labelled (neurofilament). **e** Inner and outer plexiform layers are positively stained (synaptophysin). **f** Ganglion cells and bipolar cells show positive reaction (bcl-2). **g** Neuronal cells and their cell processes are positive (NSE). **h** Nuclei of ganglion cells and bipolar cells are mostly positive, and photoreceptor cells show heterogeneous staining (Rb protein)

played heterogeneous staining. Expression of p53 protein was completely absent in the normal retina.

Retinoblastoma

Eighteen tumours contained typical rosettes of the Flexner-Wintersteiner and Homer-Wright types, which were either focal or diffusely distributed. Six were composed primarily of poorly differentiated cells with no rosette formation. Table 2 shows the immunohistochemical reactions in these. In all retinoblastomas examined, regardless of the degree of differentiation, strong NSE expres-





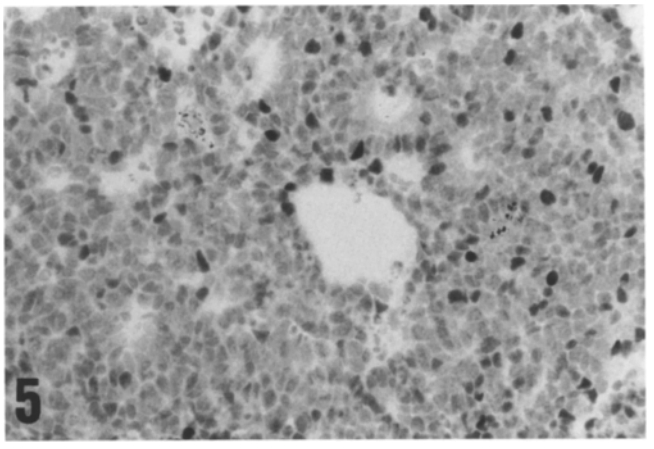


Fig. 3 Retinoblastoma. All the tumour cells are strongly labelled (NSE). ×250

Fig. 4 Retinoblastoma. Apical cytoplasm of the rosette-forming cells shows positive reaction (synaptophysin). ×250

Fig. 5 Retinoblastoma. Heterogeneous positive reaction is seen in the tumour cell nuclei (p53). ×250

sion (Fig. 3) and weak bcl-2 expression was seen in almost all the tumour cells. The overall staining pattern was very similar in differentiated and undifferentiated tumour cells. A positive reaction for synaptophysin was observed in rosette-forming cells (Fig. 4). The reaction was most intense in the apical cytoplasm immediately surrounding the lumen of the rosette, but other undifferentiated tumour cells were non-reactive. S-100-, GFAP- and vimentin-positive cells were intermingled in the perivascular areas, probably representing reactive astrocytes; the tumour cells themselves were devoid of the reactions. Neurofilament protein expression was not detected in tumour cells. Rb protein expression was completely absent in all cases examined. However, p53 protein expression was noted in 18 cases (75%), unilateral cases showing 93% (14/15) positivity and bilateral cases, 49% (4/9) positivity. Each tumour displayed marked heterogeneity in its binding to p53 protein (Fig. 5).

Discussion

The immunohistochemical profiles of 24 retinoblastomas were documented and compared with those of normal retinas by means of various immunoreagents recognizing antigens associated with a wide range of tissue types. In the normal retina, in agreement with previous results, S-100 protein, GFAP and vimentin were seen in astrocytes and/or Müller cells, and were not found in retinoblastomas [9, 14, 16]. The perivascular glial cells within the tumour showed positive reactions; tumour cells themselves were devoid of the reaction. Immunolabelling with these antigens in retinoblastoma appears to be related to astrocyte proliferation in reactive gliosis or in pre-existing astrocytes incorporated into the tumour. The pattern of immunohistochemical findings shown in this study confirmed the previously reported conclusion that the glial cells present in retinoblastomas are not neoplastic cells. Retinoblastoma in situ does not seem to have glial properties. However, neuronal cells could be responsible for the immunoreaction; neurofilament protein triplets are important cytoskeletal elements characteristic of neurons. Neurofilaments are found in retinoblastoma [16], which supports the idea of a neuronal origin. In the present study, the neurofilament antigen distribution was limited to the nerve fibre layer in the normal retina and was negative in retinoblastomas. This may be explained by the fact that our antibody detected 200-kDa and 70-kDa polypeptides but not the 160-kDa polypeptide. Neurofilament proteins have so far been shown only in a minority of tumours [11, 16] and are totally unexpressed in most cases [23].

NSE is a glycolytic enzyme essentially confined to neurons. As previously reported [6, 9, 16], we observed NSE positivity in the perikaria and cell processes of neurons in all layers of the normal retina, as well as in all cases of retinoblastoma examined, indicating that retinoblastoma has neuronal characteristics.

bcl-2 protein is associated with inhibition of apoptosis and prolonged cell survival, and is also expressed in neuronal cells of the retina [15] and in neuroblasts at all stages of differentiation [19]. Neuronal cells of the normal retina as well as almost all of the retinoblastoma cell expressed bcl-2, albeit weakly. This provides additional

support for the neuronal theory of the origin of retinoblastoma. Synaptophysin, a neuron-associated integral membrane glycoprotein of presynaptic vesicle, is concentrated in the synaptic regions of the retina, and in the rosette-forming cells of retinoblastomas [8, 12]. Existence of synaptophysin in the inner and outer plexiform layers of the retina and in retinoblastoma further supports a neuronal origin of this tumour. The immunolocalization of several photoreceptor-specific proteins has already been established, and their existence in retinoblastomas indicates photoreceptor-like differentiation [16, 20, 21]. Ultrastructurally, cells of the Flexner-Wintersteiner rosettes show photoreceptor-like differentiation [20–22]. Synaptophysin was concentrated in the apical cytoplasm of rosettes, which is thought to correspond to the outer limiting membrane of the photoreceptor rather than to their synaptic process [24]. This is in contrast to their compartmentalization in normal photoreceptor cells, and the reasons for this discrepancy remain unclear.

Inactivation of tumour suppressor genes liberates the cell from the constraints imposed by these genes, allowing unconstrained growth of the cancer cell [26]. Two such genes are the *Rb* gene and the *p53* gene, whose products control other genes, which are responsible for initiation of the cell cycle, and are involved in the regulation of cell growth [3, 4]. Rb protein expression is seen in the normal retina [2], and failure of expression in retinoblasts leads to tumour formation. As expected [17], retinoblastomas were devoid of Rb protein expression. p53 expression is seen in a variety of human malignant tumours [18], but this is the first demonstration of p53 expression in retinoblastomas. Hereditary retinoblastoma accounts for 40% of all cases and is always bilateral; nonhereditary retinoblastoma accounts for 60% and is unilateral [5]. In the present study, p53 expression showed no relation to the laterality of the retinoblastomas.

In conclusion, retinoblastoma in situ was positive for neuronal markers but negative for glial markers. Moreover, the lack of Rb protein expression and high expression of p53 protein were characteristic features.

Acknowledgements Thanks are due to Ms. Takako Akamatsu for technical assistance and to Ms. Mio Fukuchi for preparing the manuscript.

References

1. Benedict WF, Xu H-J, Hu S-X, Takahashi R (1990) Role of the retinoblastoma gene in the initiation and progression of human cancer. J Clin Invest 85:988–993
2. Cordon-Cardo C, Richon VM (1994) Expression of the retinoblastoma protein is regulated in normal tissues. Am J Pathol 144:500–510
3. Ewen ME, Sluss HK, Sherr CJ, Matsushime H, Kato J, Livingston DM (1993) Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. Cell 73:487–497
4. Finlay CA (1992) p53 loss of function: implications for the processes of immortalization and tumorigenesis. BioEssays 14:557–559
5. Gallie BL, Squire JA, Goddard A, Dunn JM, Canton M, Hinton D, Zhu XP, Phillips RA (1990) Mechanism of oncogenesis in retinoblastoma. Lab Invest 62:394–408
6. He W, Hashimoto H, Tsuneyoshi M, Enjoji M, Inomata H (1992) A reassessment of histologic classification and an immunohistochemical study of 88 retinoblastomas. A special reference to the advent of bipolar-like cells. Cancer 70:2901–2908
7. Jiang Q, Lim R, Blodi FC (1984) Dual properties of cultured retinoblastoma cells: immunohistochemical characterization of neuronal and glial markers. Exp Eye Res 39:207–215
8. Katsetos CD, Herman MM, Frankfurter A, Uffer S, Perentes E, Rubinstein LJ (1991) Neuron-associated class III β-tubulin isotype, microtubule-associated protein 2, and synaptophysin in human retinoblastomas in situ. Further immunohistochemical observations on the Flexner-Wintersteiner rosettes. Lab Invest 64:45–54
9. Kivelä T (1986) Neuron-specific enolase in retinoblastoma. An immunohistochemical study. Acta Ophthalmol 64:19–25
10. Kivelä T, Tarkkanen A (1986) S-100 protein in retinoblastoma revisited. An immunohistochemical study. Acta Ophthalmol 64:664–673
11. Kivelä T, Tarkkanen A, Virtanen I (1986) Intermediate filaments in the human retina and retinoblastoma. An immunohistochemical study of vimentin, glial fibrillary acidic protein, and neurofilaments. Invest Ophthalmol Vis Sci 27:1075–1084
12. Kivelä T, Tarkkanen A, Virtanen I (1989) Synaptophysin in the human retina and retinoblastoma. An immunohistochemical and Western blotting study. Invest Ophthalmol Vis Sci 30:212–219
13. Kyritsis AP, Tsokos M, Triche TJ, Chader GJ (1986) Retinoblastoma: a primitive tumor with multipotential characteristics. Invest Ophthalmol Vis Sci 27:1760–1764
14. Lane JC, Klintworth GK (1983) A study of astrocytes in retinoblastomas using the immunoperoxidase technique and antibodies to glial fibrillary acidic protein. Am J Ophthalmol 95:197–207
15. LeBrun DP, Warnke RA, Cleary ML (1993) Expression of bcl-2 in fetal tissues suggests a role in morphogenesis. Am J Pathol 142:743–753
16. Perntes E, Herbort CP, Rubinstein LJ, Herman MM, Uffer S, Donoso LA, Collins VP (1987) Immunohistochemical characterization of human retinoblastomas in situ with multiple markers. Am J Ophthalmol 103:647–658
17. Popoff N, Ellsworth RM (1969) The fine structure of nuclear alterations in retinoblastoma and in the developing human retina: in vivo and in vitro observations. J Ultrastruct Res 29:535–549
18. Porter PL, Gown AM, Kramp SG, Coltrera ND (1992) Widespread p53 overexpression in human malignant tumors. An immunohistochemical study using methacarn-fixed, embedded tissue. Am J Pathol 140:145–153
19. Ramani P, Lu Q-L (1994) Expression of bcl-2 gene product in neuroblastoma. J Pathol (Lond) 172:273–278
20. Rodrigues MM, Wilson ME, Wiggert B, Krishna G, Chader GJ (1986) Retinoblastoma. A clinical, immunohistochemical, and electron microscopic case report. Ophthalmology 93:1010–1015
21. Rodrigues MM, Wiggert B, Shields J, Donoso L, Bardenstein D, Katz N, Friendly D, Chader G (1987) Retinoblastoma. Immunohistochemistry and cell differentiation. Ophthalmology 94:378–387
22. Tajima Y, Munakata S, Ishida Y, Nakajima T, Sugano I, Nagao K, Minoda K, Kondo Y (1994) Photoreceptor differentiation of retinoblastoma: an electron microscopic study of 29 retinoblastomas. Pathol Int 44:837–843
23. Tarlton JF, Easty DL (1990) Immunohistological characterisation of retinoblastoma and related ocular tissue. Br J Ophthalmol 74:144–149
24. Tso MO (1980) Clues to the cells of origin in retinoblastoma. Int Ophthalmol Clin 20:191–210
25. Virchow R (1864) Die krankhaften Geschwülste, vol 2. Hirschwald, Berlin
26. Weinberg RA (1991) Tumor suppressor genes. Science 254: 1138–1146