Expression of immature (Nestin) and mature (NF & GFAP) retinal cell markers in retinoblastoma to clarify the origin of retinoblastoma

Soheir S. Shaker, *, Eman M. Ahmed*, Hala M. El Safy, * And Azza Mohamed Ahmed**

*Pathology Department, Al-Azher University, For Girls
**Ophthalmology dep. Faculty of Medicine, Ain Shams University,
Corresponding Author: soheirsaad@yahoo.com

Abstract : Despite numerous pathological studies, the origins of retinoblastomas are still controversial .***Aim:*** of this study was to clarify which factors are expressed in retinoblastoma cells and to give insights into the cell origin of retinoblastomas. **Materials and methods**: eleven patients with retinoblastoma (7 males & 4 females) patients were diagnosed in the Eye tumor Unit, Ophthalmic Department, Ain Shams University; they were divided into 5 groups according to International Classification of Retinoblastoma. Sections were examined histopathologically and immunostained by mouse monoclonal antibodies against Nestin, GFAP and NF for identification of undifferentiated stem cells, mature glial cells and mature neurons. **Results:** histopathologic findings in the examined samples revealed that 6 cases were poorly differentiated and 5 cases were well differentiated. Both Nestin and GFAP expressions were detected in the stromal cells not in the tumor cells, possibly representing muller cells and reactive stromal astrocytes respectively**,** in contrast, in all samples neurofilaments were expressed in the cytoplasm of the most retinoblastoma cells**. In conclusion**, the results of this study support that retinoblastomas are derived from mature neural cells and not originate from cancer stem cell(s).
[Soheir S. Shaker, Eman M. Ahmed, Hala M. El Safy, Azza Mohamed Ahmed. **Expression of immature (Nestin) and mature (NF & GFAP) retinal cell markers in retinoblastoma to clarify the origin of retinoblastoma.** *J Am Sci* 2013;9(1):201-208]. (ISSN: 1545-1003). http://www.jofamericanscience.org. 32

Key words: Retinoblastoma, Retinal markers [Nestin, GFAP (glial fibrillary acidic protein) and NF (Neurofilament)], Immunohistochemical analysis.

1-INTRODUCTION:

Retinoblastoma is an intraocular cancer occurring in early childhood, caused by a disruption in the tumor suppressor RB gene (Viatour and Sage, 2011). Retinoblastoma is characterized by the proliferation of cells originating from nucleated retina, it is apparent that the progression of retinoblastoma is closely related to tumor growth, with invasion of the vitreous, optic nerve, choroid, and orbit (Kopelman et al.,1987; Shields et al.,1993; Shields et al.,1994). It represents approximately 4 percent of all malignancies in children and one percent of human cancers (Shields JA, Shields, 1999).

Several methods of classification have been developed for intraocular retinoblastoma, and the more recent is the International Classification of Retinoblastoma (ICRB) (Sheilds et al., 2004; Murphree ,2005) .The current ICRB is very useful in guiding the treating clinician to choose the most appropriate treatment methods and might assist the predication of the chemoreduction and focal treatment methods success for the intraocular retinoblastoma cases (Sheilds et al.,2005; Sheilds CL, Shields, 2006).

Histologically, the tumor often consists of primitive small round cells with hyperchromatic nuclei and scanty cytoplasm (Schouten –van Meeteren, 2001). Retinoblastomas exhibit neuronal differentiation and exhibit focal regions that contain rosettes. Homer-Wright rosettes, indicative of neuroblastic differentiation are present in retinoblastoma and various neuroectodermal tumors. Flexner-Wintersteiner rosettes exhibit ultrastructural evidence of photoreceptor differentiation (Ts'o et al., 1970 a and b; David, 2008).

Despite numerous pathological studies, the origins of retinoblastomas are still controversial (Dyer and Bremner,2005). Virchow advocated that retinoblastomas arise from the glial cells of the retina. Some investigators have suggested a neuronal cell as an origin of retinoblastomas (Kivela ,1986; Kivela et al.,1989; Griegel et al.,1990;Sun et al.,1990; He et al.,1992). It has also been suggested that primitive neuroectodermal cells with their potential for both neural and glial cell lineage are the cell origin of retinoblastomas (Taylor et al., 1979; Kyritsis et al.,1984; Shuangshoti et al.,1989; Ohira et al.,1994; Mohan et al.,2006). Additionally, when cells from retinoblastoma cultures were investigated with a scanning electron microscope, glial, and neuronal cells were observed, suggesting the cell origin of the retinoblastoma is a multipotential retinal stem cell (Taylor et al,. 1979). More recent study demonstrated that retinoblastoma cells were positively stained with cancer stem cell surface markers such as ABCG2 and MCM2, supporting that retinoblastoma is derived

from stem cells (Mohan et al.,2006). Other, cells have the same origin as the photoreceptor cells (Mirshahi et al.,1986; Perentes et al.,1987; Munier et al.,1994; Nork et al.,1995).

Similar to the neurons in the central nervous system, it is generally considered that multipotential retinal stem cells give rise to all seven retinal cell types, that is, rod and cone photoreceptor cells, bipolar cells, horizontal cells, amacrine cells, Muller cells, and retinal ganglion cells, in the retinal developmental stage (Cepko et al., 1996). However, it has not been clarified whether retinoblastomas, like several types of tumours in the central nervous system, originate from such retinal stem cells.

Recent studies have disclosed that several molecules are expressed at specific times during retinal differentiation, suggesting that such molecules can be utilized as markers to specify the cell types and the degree of differentiation. Several molecules are expressed at higher levels in immature retinal stem cells compared to mature retinal cells. For example, the expression of Nestin (an intermediate filament protein expressed in stem cells) decreases upon differentiation (Tropepe et al., 2000).

On the other hand, several markers are expressed after the retinal cells are terminally differentiated. Neurofilament (NF) antigen and glial fibrillary acidic protein (GFAP) antigen are generally considered to be expressed in mature neurons and glial cells, respectively, and not in immature cells (Tropepe et al.,2000).

In this study, to clarify which factors are expressed in retinoblastoma cells and to give insights into the cell origin of retinoblastomas, we investigated the expression of the immature (Nestin) and mature (NF & GFAP) retinal cell markers in retinoblastoma specimens.

*2.*Material and methods

2.1.Materials:

2.1.1.Subjects:

The study was carried out on eleven patients with retinoblastoma (7 males & 4 females). Patients were diagnosed in the Eye tumor Unit, Ophthalmic Department, Ain Shams University, during the period from November 2009 to April 2011. Their ages ranged from 4 to 72 months. Personal and family histories with pedigree analysis were done for all cases. They were divided into 5 groups according to International Classification of Retinoblastoma cited after Salehal Al-Mesfer (2006) Table (1)

2.1.2.Samples:

Eleven primary retinoblastoma samples were obtained from enucleated eyes of all patients.

All specimens were formalin-fixed, paraffin embedded and five micron sections were prepared for routine Hematoxylin and Eosin for histopathological examination, while the other sections were mounted on positive charged slide and immunostained by mouse monoclonal antibodies against Nestin, GFAP and NF for identification of undifferentiated stem cells, mature glial cells and mature neurons respectively.

***Table(1):International classification according to* Saleh Al-Mesfer, (2006)**

International Classification of Retinoblastoma	
Group	**Features**
A	Small tumor: ≤3 mm Large tumor: >3 mm
B	Macular: ≤3 mm to foveola Juxtapapillary: ≤3 mm to disc Subretinal fluid: ≤3 mm from the marginFocal seeds
C	Subretinal seeds: ≤3 mm Vitreous seeds: ≤3 mm Both subretinal and vitreous seeds: ≤3 mm Diffused seeds
D	Subretinal seeds: >3 mm Vitreous seeds: >3 mm Both subretinal and vitreous seeds: >3 mm
E	Extensive retinoblastoma occupying more than 50% or neovascular glaucoma or opaque media from hemorrhage in anterior chamber, vitreous or subretinal space.

2.2.Methods:

2.2.1.Histopathologic evaluation

Histopathologic evaluation and scoring, by using H&E Staining was performed according to Kashyap et al., (2012) .Tumor differentiation was categorized as well differentiated (>50% Flexner-Wintersteiner rosettes) or poorly differentiated (<50% Flexner-Wintersteiner rosettes). Amount of tumor necrosis [using image analyzer optical micrometer (TSView)] estimated according to the percentage of the necrotic tumor area and was graded as none (<25%), mild (25%–50%), or extensive (>50%).

Scoring of mitosis was performed according to Schouten –van Meeteren et al. (2001). The number of mitotic figures was classified according to the number of mitoses per high power field (HPF): **Non** (fewer than 1/10 HPF), **some** (more than 1/10 HPF), **high** (more than 5/10 HPF).

2.2.1. Immunohistochemistry:

For immunohistochemical study; unstained positively charged slides (Biogenix) were prepared from each paraffin block for immunostaining with mouse monoclonal antibodies against: Nestin as a marker for undifferentiated cells (isotype IgG) [Dako, U.S.A], GFAP as a mature glial cell marker (isotype IgG) [Lab Vision neomarkers, U.S.A] and NF as a mature neuronal cell marker (isotype IgG) [Lab Vision neomarkers, U.S.A].

Immunohistochemical reactions were carried out using Labelled Streptavidin-Biotin2 System-Horseradish Peroxidase (LSAB2 System-HRP). The LSAB2 System, HRP is based on a modified labeled Avidin-Biotin (LAB) technique in which a biotinylated secondary antibody forms a complex with peroxidase-conjugated streptavidin molecules.

The entire antibody complex is made visible by addition of an appropriate substrate chromogen reagent, which is converted by the peroxidase label to brown-colored precipitate at the site of antigen localization in tissue. The chromogen used is diaminobenzidine (DAB) produced by Dako (U.S.A).

Five microns thick sections were cut from the selectively collected paraffin blocks and mounted on positively charged slides. Slides were incubated overnight at 55°C and deparaffinized. Rehydration of the slides in a graded series of alchohol at room temperature was performed. Then the slides were immersed in 3% hydrogen peroxide solution.

Slides were washed by phosphate buffer saline (PBS; pH 7.2). The mounted sections were immersed in citrate buffer (PH 6.0) then boiled in this solution in a microwave for 10 minutes. Slides were allowed to cool at room temperature then washed in PBS.

Then the slides were incubated in humidity chamber overnight with prediluted primary monoclonal antibody Nestin (recommended dilution 1:50), and with a ready to use primary monoclonal antibodies GFAP and NF.

Sections were incubated with biotinylated antimouse immunoglobulins for 10 min at room temperature, followed by washing in PBS for 5 minutes.

Then sections were incubated with streptavidin-horseradish peroxidase for another 10 minutes, followed by washing in PBS for 5 minutes. Sections were incubated with DAB for 30 minutes at room temperature. DAB was used as a color reagent.

Sections were washed in distilled water for 5 minutes then counterstained with 0.5% Mayer's hematoxylin for 1-4 minutes to enhance visualization of the tissue and cytological details. Slides were then washed with distilled water and processed in up graded alcohols. Slides were cleared in xylene for 3 minutes, and finely mount with a coverslip using DPX.Section of medulloblastoma, astrocytoma and normal cerebellum were used as positive control for Nestin, GFAP and NF respectively.

As a negative control for all markers, a tumor tissue was processed through the above sequences but the primary antibody was omitted, instead phosphate buffer solution was added.

Positive staining was indicated as brown color in the cytoplasm of the tumor cells.

2.2.3. Statistical analysis

Correlation between variables by using correlation coefficient at 0.05 significant levels was done**.**

3. Results

This study included eleven patients with retinoblastoma (7 males & 4 females). Their ages ranged from 4 to 72 months with mean age 25.6 months. One of them had family history of retinoblastoma. All cases with endophytic growth pattern. History, clinical data, and management of retinoblastoma cases are summarized in Table: (2).

Table 2 : Clinical data, and management of retinoblastoma cases

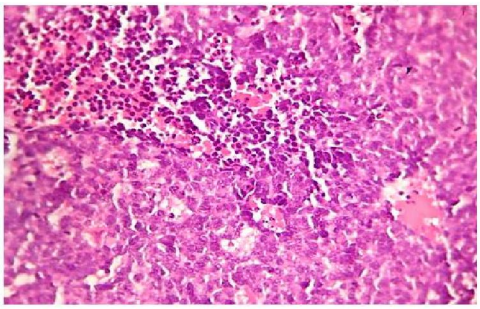
N0.	*Age (month)*	*Sex*	*Family History*	*Consanguinity*	*Laterality*	*Management*	*Group*	
							RL.	LI.
1	6	F	+ve	-ve	Bilateral	Rt.Enucleation	E	E
2	4	M	-ve	+ve (1st degree)	Bilateral	Rt.Enucleation	E	A
3	36	F	-ve	-ve	Unilateral	Rt.Enucleation	C	Free
4	24	M	-ve	-ve	Unilateral	Lt.Enucleation	Free	E
5	20	F	-ve	-ve	Bilateral	Lt.Enucleation	E	E
6	6	M	-ve	-ve	Bilateral	Rt.Enucleation	E	A
7	72	M	-ve	-ve	Bilateral	Lt.Enucleation	A	C
8	48	M	-ve	-ve	Unilateral	Rt.Enucleation	C	Free
9	36	F	-ve	-ve	Bilateral	Lt.Enucleation	E	E
10	18	M	-ve	-ve	Bilateral	Lt.Enucleation	D	E
11	12	M	-ve	-ve	Unilateral	Rt.Enucleation	E	Free

3.1.Histopathologic findings:

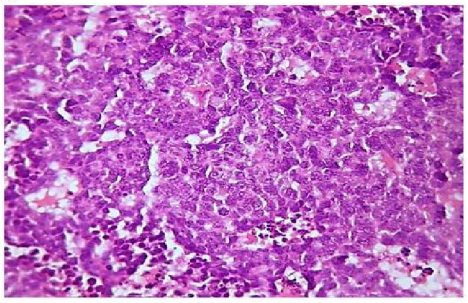
The histopathologic findings in the examined samples are summarized in Table (3). Five cases were well differentiated with the presence of Flexner Wintersteines rosettes (FW) formation more than 50% of the tumor samples (Fig. 1). The other 6 cases were poorly differentiated with presence of FW rosettes less than 50% of the tumor samples (Fig 2).

Three cases showed extensive necrotic area (> 50 %) While other 3 cases showed 25%-50% necrotic area (mild) and the residual 5 samples showed necrotic area less than 25% (none).Two cases showed (>5/10 HPF) (high) ,while mitotic figures were from (1-5/10 HPF) in 7 cases (some), and the other 2 cases showed no mitotic figures (non). Relationship between clinical and histopathologic criteria are shown in Table (4)

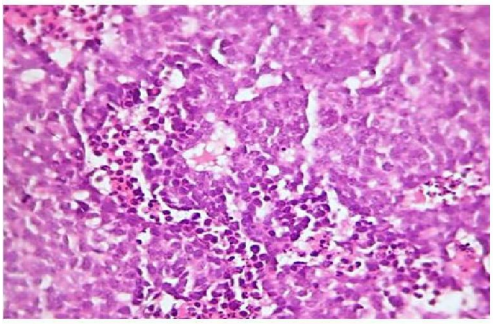
Histopatthological results



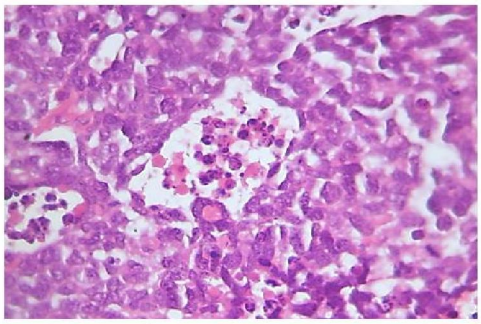
(Figure 1): Well- differentiated retinoblastoma showing numerous Flexner Wintersteines rosettes with extensive necrosis ((H &E X200)



(Figure 2) : Well- differentiated retinoblastoma showing numerous Flexner Wintersteines rosettes with mild necrosis ((H &E X200)



(Figure 3) : Poorly differentiated retinoblastoma showing few Flexner Wintersteines rosettes with extensive necrosis and mitosis ((H &E X200)



(Figure 4) : Poorly differentiated retinoblastoma showing few Flexner Wintersteines rosettes with mild necrosis ((H &E X300)

Table 3 :Histopathologic features of the examined cases of retinoblastoma

No	F.W.R	Necrotic changes	Mitotic figures
1	Poorly differentiated	Extensive	High
2	Well differentiated	Mild	Some
3	Poorly differentiated	Extensive	Some
4	Poorly differentiated	Mild	Some
5	Poorly differentiated	Non	Some
6	Well differentiated	Extensive	High
7	Well differentiated	Non	Some
8	Well differentiated	Non	Some
9	Poorly differentiated	Mild	Some
10	Poorly differentiated	Non	Non
11	Well differentiated	Non	Non

* F.W.R = Flexner Wintersteines rosettes >50%= well differentiated and <50% = Poorly differentiated

*Necrosis, none =<25% necrotic area, mild= 25%-50% and extensive=> 50%necrotic area.

*Mitosis, Non= 1/10 HPF, Some=>1 mitotic figures/10 HPF and high=> 5 mitotic figures/10 HPF.

Table (4): Relationship between clinical and histopathologic variables

Correlated variables	Correlation Coefficient	*P.* value	Degree of Correlation
Age V Differentiation	0.09	0.783*	No correlation
Age V Laterality	0.33	0.321*	Low correlation
Differentiation. V Necrosis	0.42	0.198*	Low Correlation
Differentiation. V Mitosis	0.10	0.762*	No correlation

*insignificant correlation (p. value > 0.05 at 2 tailed)

3.2.Immunohistochemistry:

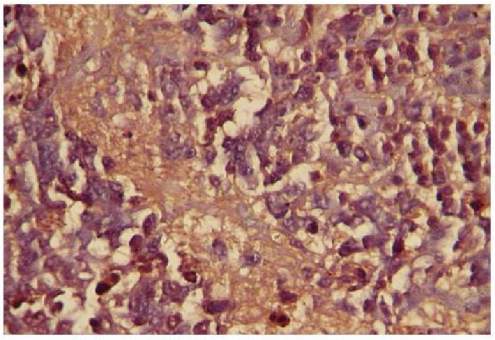
The following results for all the samples using immunohistochemistry to differentiate the origin of retinoblastoma whether cells were originated from mature neural cells or from tumor stem cells (Table5).

As a first step **Nestin** as examined. In all samples, patchy Nestin expressions were detected in the stromal cells, possibly representing Muller cells infiltrating into the retinoblastoma tissue (Figure 5). There was no expression of immature retinal cell marker (Nestin) in the retinoblastoma tissue.

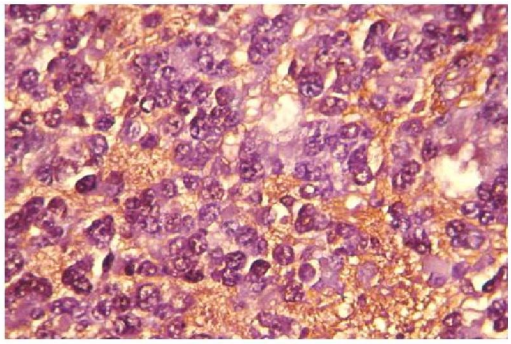
As regards GFAP, and NF, the results demonstrated that the expression of GFAP was detected only in the stromal cells not in the tumor cells, presumably representing reactive stromal astrocytes (Figure 6), GFAP expression was demonstrated in 9 out of the total 11 cases (81.8%).

In contrast, in all samples, NF was expressed in the cytoplasm of the most retinoblastoma cells (100%) (Figure 7-8).

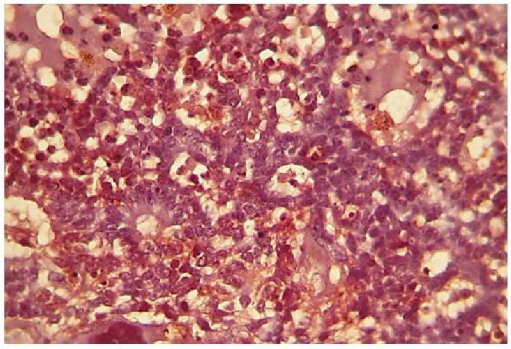
Immunohistochemical results



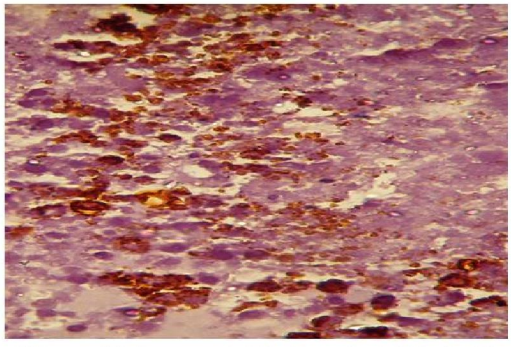
(Figure 5): Representative immunohistochemical staining for Nestin. Nestin was detected in the stromal cells not in the structure of retinoblastomas. Original magnification x 400



(Figure 6) Representativemmunohistochemical staining for GFAP. GFAP expression was detected only in the stromal cells of retinoblastomas. Original magnification x 400



(Figure 7): Representative immunohistochemical staining for NF. In all samples, NF was expressed in most of the retinoblastoma cells. Original magnification x 400



(Figure 8): Immunohistochemical staining for NF. NF was expressed in viable retinoblastoma cells with focal calcification. Original magnification x 400

Table 5:Summary of the current Immunohistochemical study

CASE NO.	Group Number		*Nestin	*GFAP	**NF
	RT. EYE	LT. EYE			
1	E	E	+	+	+
2	E	A	+	+	+
3	C	Free	+	+	+
4	Free	C	+	+	+
5	E	E	+	+	+
6	E	A	+	+	+
7	A	C	+	+	+
8	C	Free	+	-	+
9	E	E	+	-	+
10	D	E	+	+	+
11	E	Free	+	+	+

* **Nestin and GFAP** expressions were detected in interstitial cells.

****NF** expressions were detected in retinoblastoma cells.

5.Discussion

Retinal stem cells are multipotent and self renewing, expand through cell division, exit cell cycle, commit to a particular cell fate, and produce a differentiation for a particular cell type. If retinal stem cells are the origin of retinoblastoma, the tumor must express a range of retinal stem cell markers as well as differentiation markers.

Eleven patients presented with retinoblastoma were examined in this study, seven males and 4 females, with positive family history in one case and positive parental consanguinity in (9% of patients).

Histopathologic findings in the examined samples revealed that 6 cases were poorly differentiated and 5 cases were well differentiated which include one case with extensive necrosis and high mitosis. (Shields et al., 1993; Schouten-van Meeteren et al.,2001) stated that patient with poorly differentiated retinoblastoma have a worse prognosis, compared to patients with rosettes present in the tumor. Also Kashyap et al.,(2012) observed that poorly differentiated retinoblastomas present at a later age and associated with significantly more high risk factors and extensive necrosis compared with well differentiated tumors, this relationship, if correlated with a long-term follow-up, may assist in understanding the biologic behavior and progression of retinoblastoma.

A cancer stem cell population in tumors takes an essential part in tumor initiation, growth and recurrence. Cancer stem cells are thought to be responsible for chemotherapy resistance in retinoblastoma and other cancers, but the lineage of retinoblastoma remains unclear (Cassidy et al., 2012).

These cancer stem cells are immunoreactive with the stem cell markers e.g. Nestin.

Nestin is an intermediate filament (IF) protein expressed in proliferating cells during the developmental stages in a variety of embryonic and fetal tissues (Duggal and Hammond,2002). It may be involved in the organization of the cytoskeleton, cell signaling, organogenesis, cell metabolism, and represent the proliferation, migration and multi-differentiated characteristics of multi-lineage progenitor cells (Ehrmann et al.,(2005).Several immunohistochemical analyses support that several types of cell markers are detected in the retinoblastoma cells (Kyritsis et al.,1984; Shuangshoti et al.,1989; Ohira et al.,1994).

In this work an immunohistochemical study was carried out in some retinoblastoma cases to demonstrate the expression of Nestin and its coexpression with glial and neuronal IFs (GFAP & NF respectively).

The current study demonstrated no expression of immature retinal cell markers (Nestin).

Expressions of Nestin and GFAP were detected in the interstitial cells that presumably represent reactive Muller cells and reactive stromal astrocytes respectively similar to a previous study (Kohno et al.,2006; Sakata and Yanagi,2008) but not in the tumor cells.

The tumor cells were positively immunostained only with a mature neuronal cell marker, NF.

Thus, the current findings did not support the stem cell origin of retinoblastomas. On the other hand, the current results support the idea that retinoblastomas derive from postmitotic cells. In particular, the current study supports that among postmitotic cells, the origin cells are neuronal cells but not glial cells. These findings are consistent with some previous studies. For example, (Sakata and Yanagi 2008); who reported that the tumor stroma was positively immunostained only with a mature neuronal cell marker, microtubule-associated protein (MAP-2). So the retinoblastomas are derived from mature neuronal cells but do not originate from tumor stem cells.

This is in good accordance with (Sun et al., 1990) who immunostained the tumor with antibodies against GFAP, Leu-7, NSE, and Opsin, and demonstrated that NSE (mature neuronal cell marker) and Opsin are expressed in the tumor, concluding that the tumor might differentiate especially into the visual cell. (Kivela et al.,1986 and 1989); assumed the neuronal cells as the origin of the tumor because most of the cells within the tumor were immunostained with NSE.

Another immunohistochemical study demonstrated that tumor cells forming rosettes showed positive immunostaining with synaptophysin and NSE and negative immunostaining with GFAP and S-100 antigen (He et al.,1992).

Also, Cassidy et al. (2012) observed that both RB cell lines expressed all mature markers tested at the protein and mRNA levels representing several retinal cell types. This observation reinforces the potential capacity for differentiation into more mature cells.

On contrary to (Kivela et al., 1986) immunostained the retinoblastomas with antibodies against GFAP, Vimentin, NF and reported that Retinoblastoma cells were found to be devoid of all intermediate filament types studied. The tumors contained, however, vimentin and GFAP in the stromal cells. All neurofilament-positive cells in retinoblastoma apparently derived from infiltrated normal retinal areas.

On the other hand, (Seigel et al .,2007) demonstrated that subpopulations of RB cells express human embryonic and neuronal stem cell markers such as Oct3/4, Nanog, CD133, and Musashi-1. There are also subpopulations that demonstrate functional behavior (label retention and self-renewal) consistent with cancer stem cells. These findings support the hypothesis that RB is a heterogeneous tumor comprised of subpopulations with stem cell-like properties.

Further studies that include a larger number of samples, together with enhanced knowledge of yet unknown retinal cell markers, could provide us with insight into the pathogenesis of retinoblastomas and this potentially be used as target endpoints for differentiation therapies.

Acknowledgment: The Auther thank Prof. Dr. Bahaa Ghanam for valuable help and Prof. Dr. Nagwa M. Abd El Rahman for supporting the practical work . They are both professors of pathology , Faculty of medicine for girls .

5-References

1. Cassidy LL, Bejjani A, Choi M, Seigel GM (2012) :" Comparison of mature retinal marker expression in Y79 and WERI-RB27 human retinoblastoma cell line. "Oncocytology. 2:1-5.
2. Cepko CL, Austin CP, Yang X, Alexiades M, Ezzeddine D. Cell fate determination in the vertebrate retina. Proc Natl Acad Sci USA 1996; 93: 589–595
3. Duggal N and Hammond RR (2002): ‘’Nestin Expression in Ganglioglioma’’ Experimental Neurology., 174: 89-95.
4. David MacPherson: Insights from mouse models into human retinoblastoma. Cell Division 2008, 3:9 doi:10.1186/1747-1028.
5. Dyer MA, Bremner R. The search for the retinoblastoma cell of origin. Nat Rev Cancer 2005; 5: 91–101.
6. Ehrmann J, Kola'r Z and Mokry J (2005): ‘’Nestin as a diagnostic and prognostic marker: immunohistochemical analysis of its expression in different tumours’’ J Clin Pathol., 58: 222-223.
7. Griegel S, Heise K, Kindler-Rohrborn A, Rajewsky MF. In vitro differentiation of human retinoblastoma cells into neuronal phenotypes. Differentiation 1990; 45: 250–257.
8. He W, Hashimoto H, Tsuneyoshi M, Enjoji M, Inomata H. A reassessment of histologic classification and an immunohistochemical study of 88 retinoblastomas. A Special Reference to the Advent of Bipolar-Like Cells. Cancer 1992; 70: 2901–2908
9. Kashyap S, Sethi S, Meel R, Pushker N, Sen S, Bajaj MS, Chandra M, Ghose S (2012) : " Histopathologic analysis of eyes primary enucleated for advanced intraocular retinoblastoma from a developing country. Arch Pathol Lab Med. 2012 ;136(2):190-3
10. Kivela T, Tarkkanen A, Virtanen I. (1986): "Intermediate filaments in the human retina and retinoblastoma" Investigative Ophthalmology & Visual Science 27, 1075-1084
11. Kivela T. Neuron-specific enolase in retinoblastoma. An immunohistochemical study. Acta Ophthalmol (Copenhagen) 1986; 64: 19–25.
12. Kivela T, Tarkkanen A, Virtanen I. Synaptophysin in the human retina and retinoblastoma. An Immunohistochemical and Western Blotting Study. Invest Ophthalmol Vis Sci 1989; 30: 212–219.
13. Kopelman JE, McLean IW, Rosenberg SH. Multivariate analysis of risk factors for metastasis in retinoblastoma treated by enucleation. *Ophthalmology*. 1987;94:371–377.
14. Kohno H, Sakai T, Kitahara K (2006): Induction of nestin, Ki-67, and cyclin D1 expressionin Muller cells after laser injury in adult rat retina. Graefes Arch Clin Exp Ophthalmol; 244: 90–95.
15. Kyritsis AP, Tsokos M, Triche TJ, Chader GJ. Retinoblastoma – origin from a primitive neuroectodermal cell? Nature 1984; 307: 471–473.
16. Mirshahi M, Boucheix C, Dhermy P, Haye C, Faure JP. Expression of the photoreceptor-specific S-antigen in human retinoblastoma. Cancer 1986; 57: 1497–1500.
17. Munier FL, Balmer A, van Melle G, Gailloud C. Radial asymmetry in the topography of

retinoblastoma. Clues to the Cell of Origin. Ophthalmic Genet 1994; 15: 101–106.
18. Murphree AL. Intraocular retinoblastoma: the case for new group classification. Ophthalmol Clin North Am 2005;200(viii):41-53.
19. Mohan A, Kandalam M, Ramkumar HL, Gopal L, Krishnakumar S. Stem cell markers: ABCG2 and MCM2 expression in retinoblastoma. Br J Ophthalmol 2006; 90: 889–893.
20. Perentes E, Herbort CP, Rubinstein LJ, Herman MM, Uffer S, Donoso LA et al. Immunohistochemical characterization of human retinoblastomas in situ with multiple markers. Am J Ophthalmol 1987; 103: 647–658.
21. Nork TM, Schwartz TL, Doshi HM, Millecchia LL. Retinoblastoma. Cell of Origin. Arch Ophthalmol 1995; 113: 791–802.
22. Ohira A, Yamamoto M, Honda O, Ohnishi Y, Inomata H, Honda Y. Glial-, neuronal – and photoreceptor-specific cell markers in rosettes of retinoblastoma and retinal dysplasia. Curr Eye Res 1994; 13: 799–804.
23. Saleh Al-Mesfer, MD (2006): :"International classification and management of retinoblastoma" Saudi Journal of ophthalmology 20,161-162.
24. Sakata R and Yanagi Y (2008): "Expression of immature and mature retinal cell markers in retinoblastoma" Eye 22, 678-683.
25. Seigel GM, Hackam AS, Ganguly A, Mandell LM, Gonzalez-Fernandez F (2007): "Human embryonic and neuronal stem cell markers in retinoblastoma" Molecular Vision 13, 823-832.
26. Shields CL, Shields JA, Baez KA, Cater J, De Potter PV. Choroidal invasion of retinoblastoma: metastatic potential and clinical risk factors. *Br J Ophthalmol.* 1993;77:544–548.
27. Shields CL, Shields JA, Baez KA, et al. Optic nerve invasion of retinoblastoma: metastatic potential and clinical risk factors. *Cancer.* 1994;73:692–698.
28. Shields JA, Shields CL. Retinoblastoma. In: Shields JA, Shields CL, editors. Atlas of intraocular tumors. Philadelphia: Lippincott Williams; 1999.pp.207-232.
29. Sheilds CL, Mashayekhi A, Demirci H, et al. A practical approach to management of retinoblastoma. Arch ophthalmol 2004;122:729-735.
30. Sheilds CL, Au Ak, Czyz C, et al. The International Classification of Retinoblastoma (ICBR) predicts chemoredction success. Presented at the International Society of Ocular Oncology; September 1-5, 2005; Whistler, Canada and American Academy of Ophthalmology October 14-16, 2005.
31. Sheilds CL, Shields JA. Basic understanding of current classification and management of retinoblastoma. Curr Opin Ophthalmol 2006;17:228-234.
32. Schouten-Van Meeteren A.Y.,VALK P., LIN-DEN H.C and MOLL A.C.: Histopathologic features of retinoblastoma and relation with in vitro drug resistance measured by mean of the MTT assy .cancer,92 (11):2933-2940.2001.
33. Sun XL, Yokoyama T, Minoda K, A S. Immunohistochemical studies of retinoblastoma. Jpn J Ophthalmol 1990; 34: 149–157.
34. Shuangshoti S, Chaiwun B, Kasantikul V. A study of 39 retinoblastomas with particular reference to morphology, cellular differentiation and tumor origin. Histopathology 1989; 15: 113–124.
35. Taylor HR, Carroll N, Jack I, Crock GW. A scanning electron microscopic examination of retinoblastoma in tissue culture. Br J Ophthalmol 1979; 63: 551–559.
36. Ts'o MO, Fine BS, Zimmerman LE: The nature of retinoblastoma. II. Photoreceptor differentiation: an electron microscopic study. *Am J Ophthalmol* 1970 a, 69:350-359.
37. Ts'o MO, Zimmerman LE, Fine BS: The nature of retinoblastoma. I. Photoreceptor differentiation: a clinical and histopathologic study. *Am J Ophthalmol* 1970 b, 69:339-349.
38. Tropepe V, Coles BL, Chiasson BJ, Horsford DJ, Elia AJ, McInnes RR et al. Retinal stem cells in the adult mammalian eye. Science 2000; 287: 2032–2036.
39. Viatour P, Sage J. Newly identified aspects of tumor suppression by RB. Dis Model Mech 2011; 4(5): 581-5.

12/29/2012