

Research Article

Multi-drug Resistant Proteins Expression in Retinoblastoma in Primary Enucleated Eyes *Versus* Eyes Enucleated after Failure of Conservative Treatment

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Abstract

Objective: To compare expression of Multidrug-resistant protein 1/P-glycoprotein (MDR1/Pgp) in retinoblastoma in eyes treated by primary enucleation due to advanced tumor at initial presentation and those enucleated after being resistant to chemotherapy.

Design and methods: A prospective non randomized masked analysis of pathology specimens obtained from twenty retinoblastoma patients presenting at the retinoblastoma clinic at ophthalmology department, Ain Shams University Hospitals. Specimens from patients who had enucleation were divided into 2 groups. Patients in group 1 underwent primary enucleation due to advanced tumor at presentation. Patients in group 2 underwent secondary enucleation after failure of conservative treatment. Immuno histochemical studies were performed searching for expression of Multidrug-resistant protein 1/P-glycoprotein (MDR1/ Pgp) in the two groups. Patient demographic and eye examination data we collected and reviewed.

Results: Analysis of the primary enucleation group showed high positive, low positive and negative expression in 1 (10%), 2 (20%) and 6 cases (70%) respectively. In secondary enucleation group 5 cases (50%), 3 cases (30%) and 2 cases (20%) showed high positive, low positive and negative expression respectively.

Conclusion: This pilot study though, not being able to demonstrate statistical significance in MDR1 expression in primary enucleated vs. secondary enucleated resistant cases, demonstrated p value low enough to indicate a trend for more MDR1 expression in resistant cases (p=0.068). Further study with a larger sample size is warranted.

Keywords: MDR protein-1; P-glycoprotein; Retinoblastoma; Tumor; Chemotherapy

Introduction

Retinoblastoma is the commonest primary intra-ocular malignant neoplasm of childhood [1]. It accounts for about 3% of all childhood cancers. It occurs in about 1:17000 live births [2].

Though potentially curable, untreated cases typically die of their tumor 2–4 years after onset [1,3].

Whilst contained in the eye, treated retinoblastoma survival rates exceed 95% and decrease below 50% with extra-ocular spread [4].

Dramatic regression with nitrogen mustard chemotherapy combined with radiation for retinoblastoma was first reported in 1953 [5].

Primary systemic chemotherapy (chemoreduction) became in the1990s a mainstay of retinoblastoma treatment. Combined with a variety of consolidating focal treatment modalities, chemotherapy makes retinoblastoma more treatable carrying less risk of damaging vision [6-8] avoiding enucleation and avoiding external beam radiation with its complications [9].

Noticeable treatment responses are observed in more than 90% of eyes; being maximum after 2 cycles [10-12].

On the contrary chemotherapy alone can only save less than 10% of the eyes [10]. Up to two-thirds of macular tumors can be controlled with chemotherapy alone probably because of the richer vascular supply maximizing exposure to chemotherapy [13,14].

However, many tumors prove resistant. Drug resistance can occur at multiple levels: host drug metabolism, drug delivery, microenvironment, and cellular mechanisms [15,16]. Tumors may show resistance after initial good response [17].

ATP binding cassette (ABC) transporters are ATP-dependent membrane proteins. They are expressed in tissues throughout the body, especially in organs such as the liver, intestine [18]. They are extensively involved in transport of drugs and substrates across cell membrane and their metabolism [19].

Chan et al. were the first to report on increased expression of MDR1/ Pgp, an ABC transporter, in retinoblastoma [20,21]. Chan et al. further reported improved chemotherapy for retinoblastoma outcomes in patients in whom chemotherapy was supplemented with cyclosporine, an MDR1/ Pgp inhibitor [22].

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Page 2 of 6

Purpose

This study was designed to try to compare expression of Multidrugresistant protein 1/P-glycoprotein (MDR1/Pgp) in retinoblastoma in eyes treated by primary enucleation due to advanced tumor at initial presentation and those enucleated after being resistant after being subjected to chemotherapy.

Methods

This study was a prospective study. Twenty retinoblastoma patients presented to the ocular oncology clinic at ophthalmology department, Ain Shams University Hospitals. All patients had enucleation as described by Nunery et al. 2012 with minor modifications [23]. They were divided into 2 groups. Group 1 includes patients undergoing primary enucleation due to advanced disease, presenting with either of Glaucoma. anterior chamber tumor, suspected optic nerve involvement (clinically or radiological), orbital extension or large tumor involving more than 1/2 of the retina with poor visual potential group 2 included those with secondary enucleation after failure of conservative treatment as indicated by a new onset of glaucoma, anterior chamber or optic nerve involvement or orbital extension while on chemotherapy or a continued clinical tumor growth after 2 chemotherapy cycles in eyes with a tumor filling >2/3 of vitreous cavity or <2/3 of vitreous cavity but with 2 local treatment cycles (laser photocoagulation or cryotherapy) or a tumor showing extensive vitreous seeding.

Patients were subjected to detailed history, and eye examination including pupils assessment, external examination, slit lamp examination (if possible), dilated fundus examination (under general anesthesia using tropicamide 1% drops) and fundus photography. An US, CT scan or MRI were done. Chemotherapy regiment included monthly cours of the drugs: Vincristine, Carboplatin, and Etoposide (V P 16).

All enucleated eyes were preserved in formalin. Histopathological examination was documented after conventional Haematoxylin and Eosin (H&E) staining. Immunohistochemical studies for detection of Multidrug-resistant protein1/P-glycoprotein.

Paraffin sections were deparaffinized in xyeline and rehydrated in graded series of ethanol, then washed in phosphate buffer saline (PBS) PH 6.7 for 10 minute. Positive control slides were prepared from / tissue Breast canrcinoma. Negative control slides were prepared from the same tissue block as the specimen and instead of using a primary antibody, negative control serum was applied (Biogenex laboratories). DAB "2,3 diamino-benzidine" forms brownish end product that is insoluble in alcohol and, therefore is suitable for permanent mounting. It is prepared by adding 2 drops of liquid DAB chromogen to one vial of 2.5 ml ready-to-use substrate buffer, then adding 1 drop of hydrogen peroxide (H₂O₂) substrate buffer solution and mix well. It should be freshly prepared "10–15 minutes before use". Staining Procedures was done at room temperature using immunostainer (Shandon Sequenza Immunostainer) to avoid dryness of tissue sections. Application of peroxidase block was done by application of 3% H₂O₂ in water to the tissue sections and incubated for 5-10 minutes at room temperature, then rinsed by PBS pH 7.6 for 5 minutes.

Application of primary antibody was done by addition of 1-2 drops of primary antibody to the tissue sections then incubated for the recommended time. For p-glycoprotein (p170), tissue sections were incubated for "30" minutes at room temperature. Enough amount of Multi-link (1-2 drops) was added to cover each section and incubated for 20 minutes, then rinsed by PBS for 5 minutes. Application of Label was done by addition of appropriate volume of label to cover the specimen and incubated for 20 minutes, then rinsed by PBS for 5 minutes. Counter staining procedure by immersing the slides in a bath of Mayer's hematoxylin for 1-3 minutes. The slides were rinsed with distilled water. Dehydration in 95% ethanol, followed by absolute ethanol, clearing of the slides by xylene using 2 baths, mounting of the cover slip using 1-2 drops of mounting media (Canada Balsam) was done.

Sections were examined by ordinary light microscope. Evaluation of p-glycoprotein (p170) stain and positive results were considered as brown membranous immunostaining. Statistical analysis for demographic data analysis included age at presentation, age at enucleation and presentation–enucleation interval, gender and laterality of the tumor. Histopathological data analysis was done analyzing tumor differentiation, optic nerve invasion and choroidal infiltration. Immuno-histochemical analysis was done for MDR1.

Quantitative data ware presented as mean \pm standard deviation. Independent t-test was used to compare the means of two groups. Qualitative data ware presented as count and appropriate proportion. Chi-square test and/ or Fisher exact test was used to compare the two independent proportions. Significant results were considered with $p \le 0.05$. However, a p value <0.10 and >0.05 should be viewed as suggesting a true difference that may be masked by the relatively small number of cases. SPSS (Statistical Package for Social Sciences) version 22.0 was used in data entry and analysis. (IBM Corporation, 2013).

Results

Table 1 shows the characteristics and findings of cases included in the study. In group 1 the age of presentation ranged from 8 months up to 72 months with a mean of 32.4 months (\pm 21.36), the age of enucleation ranged from 14 months up to 72 months with a mean of 34.3 months (\pm 20.74) and presentation-enucleation interval showed a mean of 1.90 months.

Regarding group 2, the age of presentation ranged from 8 months up to 75 months with a mean of 29.2 months (\pm 24.06), the age of enucleation ranged from 19 months up to 96 months with a mean of 53.9 months (\pm 28.10) and presentation-enucleation interval showed a mean of 24.70 months. P-values were 0.757, 0.094, 0.004 for age of presentation, enucleation and presentation-enucleation interval respectively, being non-significant except for presentation-enucleation interval (Figure 1).

Case	Por R	IHC	Sex	Age at enucleation in months	Age at 1st diagnosis in months	Presentation - enucleation interval in months	Laterality	Grading	ON invasion	Choroidal infiltration
1	R	low +ve	м	96	75	21	В	Moderate	-ve	-ve

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Page	3	of	6

2	Р	-ve	F	29	29	zero	В	Moderate	+ve	-ve
3	Р	-ve	F	72	72	zero	В	Moderate	+ve	-ve
4	R	low +ve	м	96	72	24	В	extensive necrosis	+ve	+ve
5	R	High +ve	F	19	8	11	В	Moderate	-ve	-ve
6	Р	-ve	м	12	12	zero	U	well differentiated	-ve	-ve
7	R	High +ve	М	24	21	3	В	Moderate	-ve	-ve
8	Р	low +ve	м	17	8	9	В	well differentiated	-ve	-ve
9	Р	-ve	F	42	41	1	U	Moderate	-ve	+ve
10	R	High +ve	М	36	16	20	В	Moderate	+ve	+ve
11	Р	High +ve	F	36	33	3	В	Moderate	-ve	+ve
12	Р	-ve	F	65	62	3	U	Moderate	-ve	+ve
13	R	High +ve	М	72	14	58	U	Poor	+ve	-ve
14	R	-ve	М	48	24	24	U	Moderate	-ve	-ve
15	R	High +ve	F	29	25	4	U	Poor	-ve	-ve
16	Р	-ve	м	36	35	1	В	Moderate	+ve	+ve
17	Ρ	High +ve	F	14	13	1	U	well differentiated	-ve	-ve
18	R	-ve	М	53	25	28	В	Poor	+ve	+ve
19	Р	-ve	F	20	19	1	U	Moderate	-ve	-ve
20	R	High +ve	F	66	12	54	В	well differentiated	-ve	-ve

Table 1: Characteristics and Findings in different cases of retinoblastoma (P: Primary Enucleation; R: Resistant Enucleation; +ve: Positive, -ve: Negative; M: Male, F: Female; B: Bilateral Retinoblastoma; U: Unilateral Retinoblastoma)

Seven cases (70%) of group 1 were females, while 7 cases (70%) of group 2 were males. Regarding tumor laterality, bilateral Tumors were found in 5 cases (50%) of primary enucleation group and 3 cases (30%) of group 2. P-values were insignificant for both sex and laterality, being 0.074 and 0.325 respectively.

Comparison between the two groups regarding histopathological differentiation was made. Regarding tumors of primary enucleation; 3 (30%), and 7 (70%) were well-differentiated, and moderately differentiated respectively. One (10%), 5 (50%) and 3 cases (30%) of secondary enucleation eyes had well, moderate, and poorly differentiated tumors, respectively. One tumor (10%) was extensively necrotic and non- gradable. There was no statistically significant difference between the two groups. P value was 0.184.

Of the 10 eyes with primary enucleation, 3 tumors (30%) showed optic nerve invasion and 4 Tumors were positive for Choroidal infiltration. This contrasts with 4 Tumors (40%) and 3 Tumors (30%) respectively in the secondary group. P value was 1 for both, being insignificant.



Figure 1: Comparing the primarily and secondarily enucleated eyes regarding the interval between presentation to the hospital and patient/ legal guardian consent followed by enucleation.

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A comparison between the two groups regarding expression of MDR1 was made. Tumors expression was graded as high positive if more than half cells express MDR1 (Figure 2a), low positive, if less than half cells express the protein (Figure 2b) or negative (Figure 2c).

Regarding primary enucleation group, 1 case (10%) showed high positive expression, 2 cases (20%) showed low positive expression and 7 cases (70%) showed negative expression. Expression in secondary enucleation group was 5 cases (50%), 3 cases (30%) and 2 cases (20%) for high positivity, low positivity and negative expression respectively. There was no statistically significant difference between the two groups. P value was 0.068.

A comparison between immune histochemistry (IHC) positive and IHC negative cases were made regarding demographic data, tumor laterality and histopathological features. For IHC positive group the age of presentation ranged from 8 to 75 months with a mean of 27 months (\pm 24.16), the age of enucleation ranged from 14 to 96 months with a mean of 45.95 months (\pm 31.05) and presentation-enucleation interval showed a mean of 18.91 months. Regarding immuno-negative cases, the age of presentation ranged from 12 to 72 months with a mean of 35.44 months (\pm 19.92), the age of enucleation ranged from 12 to 72 months with a mean of 41.89 months (\pm 19.92) and presentation-enucleation interval showed a mean of 6.44 months. P-values were 0.41, 0.74, 0.11 for age of presentation, enucleation and presentation-enucleation interval respectively, all are insignificant.

Six cases (54.5%) of MDR1 positive cases were males, while 4 cases (44.4%) of negative cases were males. Regarding tumor laterality, bilateral Tumors were found in 8 (72.7%) of positive and 5 (55.6%) of negative expression cases. P-values were insignificant for both sex and laterality, being 1 and 0.362 respectively.

Comparison between the two groups regarding histopathological differentiation was made. Regarding tumors with positive staining; 3 cases (27.3%) were well-differentiated, 5 cases (45.5%) were moderately differentiated, 2 cases (18.2%) were poorly differentiated and 1 case (9.1%) showed extensive necrosis with inability to grade the differentiated, 7 cases (77.8%) were moderately differentiated, 2 cases (20%) had poorly differentiated tumor and one tumor (11.1%) was poorly differentiated. There was no statistically significant difference between the two groups. P value was 0.564.

Of IHC positive cases, 3 tumors (27.3%) were positive for each of optic nerve invasion and choroidal infiltration. 4 Tumors (44.4%) were positive for both in the IHC negative cases. P value was 0.37 for both this difference was insignificant.

Discussion

Retinoblastoma remains an important tumor of childhood. While, introduction of systemic chemotherapy as a treatment modality saved, many eyes from enucleation, many tumors resist chemotherapeutic agents necessitating enucleation.

Why some tumors respond to chemotherapy and some do not remains an important question to be answered. A hypothesis was suggested that multidrug resistant proteins are the major cause or at least in part, for tumor resistance. To explain for the failure of chemotherapeutic regimens containing cyclosporin, Chan et al. in a later study suggested that multidrug resistance associated protein-1 (MRP1), another ABC transporter, conveys an alternative means of drug resistance in the presence of MDR1/Pgp inhibitors.



Figure 2: MDR/ Pgp immunostaining of 3 speciemens from enucleated eyes; A: (upper)demonstrates high positive staining, B: (middle) demonstrating low positive staining, C:(lower) demonstrating negative staining.

They found that 3 out of 3 retinoblastomas which were refractory to treatment expressed this transporter. This contrasts with the low level

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Page 5 of 6

of expression found in primary enucleated tumors, being 1 out of 18 retinoblastomas [24].

Krishnakumar et al. investigated expression of P-gp, the product of MDR1 gene, in 60 retinoblastomas. All were subject to primary enucleation. In this cohort, 27 tumours had no invasion of choroid, optic nerve or orbit and 33 tumours had a positive invasion [25].

Among the 60 tumours P-gp was expressed in 23 (38%) tumours. P-gp was expressed in 11/27 (40%) tumours with no invasion and in 12/33 (36%) tumours with invasion. There was no correlation between P-gp with invasion, differentiation and laterality of the tumours and response to post-operative chemotherapy [26].

In 2005, Filbo et al. compared expression of P-gp in eyes enucleated as a primary treatment and those enucleated after failure of chemotherapy. They also correlated their presence with the degree of tumor differentiation in a semiquantitative manner. They found that Pgp is expressed in 60% of primary enucleated eyes compared to 66.6% in the other group which all had high levels of expression. P-gp was expressed in 81.2% of well-differentiated tumors [27].

They concluded that P-gp is more expressed in well-differentiated tumors especially those tumors which failed chemotherapy. They suggested this as a possible cause of treatment failure [28].

Wilson et al. investigated P-gp expression in primary enucleated eyes. Reporting a low expression level, being found in only 2/16 retinoblastomas eyes (12%) [26].

We tried to verify this hypothesis in this study. Our proposal was to first measure the presence and intensity of expression of MDR1 in enucleated tumors that were resistant to chemotherapy from the start. Also to check for tumors, showing, during chemotherapy, modification of cellular carcinogenic characteristics. This change in characteristics may induced by the chemotherpy Vincristine, Carboplatin, and Etoposide (V P 16). This appears to the ophthalmologist as tumors that grow in spite of chemotherapy course of treatment and compare these with the primary enucleated tumors which suggest that they are aggressive from the start.

The ideal situation for testing if chemotherapy in some tumor population induces increase in tumor resistance to chemotherapy wholly or partially by inducing increased expression of MDR1. The ideal situation is to test this in patients who were diagnosed with bilateral retinoblastoma. With a very advanced staging in one eye needing enucleation which when done we measure the MDR 1 expression in the enucleated tumor tissue and the patient takes chemotherapy for the other eye and the tumor continues to grow needing a second eye enucleation. The excised tissue is test for MDR1 and we compare the specimens from the same patients that were subjected to chemotherapy and that tumor that was not; thus having a precise evaluation of the effect of chemotherapy on increased tumor resistance to chemotherapy. This ideal bilateral speciemen is not, and should not, be common. A second choice, though not the best was to compare treated cases (Stage B and C) with stage D and E cases who were enucleated at presentation and tested for immunohisto staining for MDR1.

This study did not demonstrate a clear cut difference in expression of MDR1 between primary enucleated eyes and secondary enucleated eyes. As the P-value for this difference was 0.068, though being nonsignificant, the value is low enough to indicate a trend for more MDR1 expression in resistant cases. With the possibility of acquired resistance during the course of chemotherapy. The value may reach significance if the sample size were to be increased.

In comparison to these findings, in 2004, Krishnakumar et al. worked only on primary tumors investigating expression of MDR1 in 60 tumors. They showed expression in 23 eyes (38%). This figure is very near to that in our study (30%). They showed that expression is not related to laterality, differentiation, invasion or response of metastatic tumor to post-enucleation chemotherapy [24].

Filho et al. found P-gp expression to be very similar in two groups of primary and secondary enucleation, the percentage of expression was 60% and 66.6% respectively. All secondary enucleated eyes positive for P-gp showed high expression [25].

On the other hand, MDR1 expression was clearly not related to age at presentation, age at enucleation, presentation-enucleation interval, gender, tumor laterality or histopathological features as tumor differentiation, optic nerve invasion or choroidal infiltration.

Worth of note, in this study, is the delayed age of presentation of retinoblastoma compared to that documented in international literature. While the average age of presentation in literature is 24 months for unilateral diseases and 9 -12 months for bilateral diseases [28], in our study it was 26.25 months for unilateral cases and 33.8 months for bilateral cases.

This study was not free of shortcomings; one was the small sample size which may have prevented some data from reaching statistical significance. A future study can compare MDR1 expression in both eyes in the same patient if primary enucleation was indicated in one eye and the other had to be enucleated after failure of chemotherapy. Confirmed new appearance or increase in MDR1/ Pgp expression during in resistant tumors may point at the cause of resistance.

Conclusion

This study demonstrated both a higher percentage and greater degree of MDR1/ Pgp expression among tumors subjected to enucleation after failing chemotherapeutic agents, this difference was approaching but not reaching clinical significance.

The number of cases in our series limited our ability to confirm the relationship between resistance and expression. A study with larger number of cases may increase confidence of our findings.

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Page 6 of 6