Association between P-glycoprotein and Multi-Drug Resistance Associated Protein 1 Expression and Clinical Outcomes in Iranian Retinoblastoma

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Abstract

***Purpose*:** The aim of the present study was to detect the expression of P-glycoprotein (P-gp) and multi-drug resistance associated protein 1 (MRP1) in retinoblastoma tumors, and analyze the relationship between its expression, the clinico-pathological features and recurrence.

***Methods*:** P-gp and MRP1 expression in formalin-fixed paraffin-embedded retinoblastoma bearing ocular tissue sections from 26 patients were studied by immunohistochemistry assay, and the results were analyzed in correlation with clinico-pathological parameters. Patients were classified into two groups according to their treatment: with (n=16) and without (n=10) preoperative chemotherapy.

***Results*:** Six (23%) and 12 (46%) tumors were P-gp and MRP1 positive respectively. Three out of five tumors that presented P-gp had tumor invasion while MRP1 was positive in four tumors with invasion. Seven patients showed recurrence during follow-up time that P-gp and MRP1 were expressed in three and four of them, respectively. There was no correlation between protein expression and clinical outcomes as recurrence in this study.

***Conclusion*:** MRP1 and P-gp were intrinsically expressed in our study population. However, the results did not demonstrate significant association between P-gp and/or MRP1 positive expression and unresponsiveness to chemotherapy in Iranian retinoblastoma patients.

***Keywords*:** Chemotherapy, Multi-Drug Resistance Associated Protein 1, P-glycoprotein, Retinoblastoma

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Introduction

Retinoblastoma is a common childhood malignancy that arises from retinal sensory cells, and affecting 1:14000-1:20000 live newborns.[1] The broad range of mortality rates has been observed among different regions of the world. Due to advanced stages of the tumor at the presenting time, our mortality rate from this malignancy is higher than developed countries.[2,3] Current treatments for retinoblastoma are enucleation, systemic and intra-arterial chemotherapy for massive tumors and cryotherapy, laser therapy, and plaque radiotherapy for small tumors.[4-6] Chemotherapy has been found to be the critical treatment option for this disease, since it is effective way for salvage of the affected eye.[5,6] Resistance to chemotherapy is the major obstacle, limiting the success of cancer treatment.

ATP-Binding Cassette (ABC) transporters were found to associate to chemo-resistance by the energy-dependent efflux of anticancer agents from tumor cells. The multi-drug resistance 1/P-glycoprotein (MDR1/P-gp) and multi-drug resistance associated proteins 1 (MRP1) are two common members of the human ABC transporter to mediate multidrug resistance in cancer therapy. These proteins are physiologically expressed in the several normal tissues, such as the kidney, intestine, colon, adrenal glands, and capillary endothelium of the brain and testes to expel exogenous toxins and endogenous metabolites.[7,8] In addition, these proteins pump many anticancer drugs out of the tumor cells, and confer drug resistance by decreasing their intracellular concentrations.[9-12]

Chan et al[13] were the first to demonstrate the multidrug-resistance in human retinoblastoma cell lines. Furthermore, high expression of MDR1/P-gp was reported in five chemo-resistant cells lines.[14] On the other hand, Chan et al[15] showed that the use of cyclosporin A, an inhibitors of P-gp, in combination with chemotherapy improves clinical outcomes in retinoblastoma patients. MRP1 protein, however, might cause a different status of drug resistance in this disease despite the use of cyclosporine.[16]

Vincristine, etoposide, and carboplatin (VEC) as the drugs used most often for retinoblastoma treatment, were identified as substrates for some of the most commonly studied ABC transporters in drug resistance. A clinical study of these proteins in retinoblastoma patients is needed. To date, there is no report about the expression of ABC transporters in retinoblastoma from Iranian children. Herein, the expression of the P-gp and MRP1 proteins in retinoblastoma tumors of enucleated eyes and their correlation with the clinico-pathological parameters were studied.

Methods

Study population and treatment

The study was approved by the ethics committee of Tehran University of Medical sciences. Paraffin embedded tissue blocks from 26 enucleated eyes with retinoblastoma tumor between Jan 2009 and Jan 2011 were retrieved. Clinico-pathological characteristics were obtained from medical records and surgical pathology reports. Sixteen out of 26 patients received pre-enucleation chemotherapy (VEC) and the rest were managed by primary enucleation for stage E disease. The patients with different protocol of treatment were excluded from the study.

Immunohisthochemistry

Four-micrometer thick sections of paraffin embedded tumors on the slides and incubated for 30 min at 60°C. Sections were routinely deparafinized in xylene and rehydrated in a graded ethanol series. Endogenous peroxidase activity was blocked by incubation in 0.3% H_2O_2 for 30 min. Antigen retrieval was achieved by heating the tissue sections in an autoclave in sodium citrate buffer for 10 min at 120°C. After cooling at room temperature, the sections were rinsed in phosphate-buffered saline (PBS, PH=7.4). The sections were incubated with primary antibodies, anti-P-gp [JSB-1, 1:40, ab3366, Abcam, Cambridge, UK) for 1 h and anti-MRP1 (MRP-r1, 1:20, ab3368, Cambridge, UK)] for 2 h and rinsed in PBS.

The sections were subsequently treated with secondary antibody, EnVision+ Dual Link System-HRP (K4061, Dako, Copenhagen, Denmark) for 45 min and rinsed with PBS. Immunoreactivity was visualized with 3,3 diaminobenzidine (Dako, Copenhagen, Denmark). The sections were counterstained

with hematoxylin and mounted. Normal colorectal tumor tissues were used as positive control for both proteins (Figure 1). The primary antibody was replaced by PBS in negative controls. Positive and negative controls were applied in each run of the procedure. Slides were scored by an experienced ocular pathologist (FAA) who was blinded to drug responses. Samples were classified in two groups, positive or negative, as reported in a previous report.[17]

Statistical analysis

Data were analyzed using the SPSS version 11.0 (SPSS, Inc., Chicago, IL). Significant p-value considered as less than 0.05. Relationship between clinico-pathological parameters, drug response, and IHC staining of P-gp and MRP1 were determined using χ^2 test. Kaplan-Meier survival analysis was used for survival analyses and Log Rank test was used for comparing survival data.

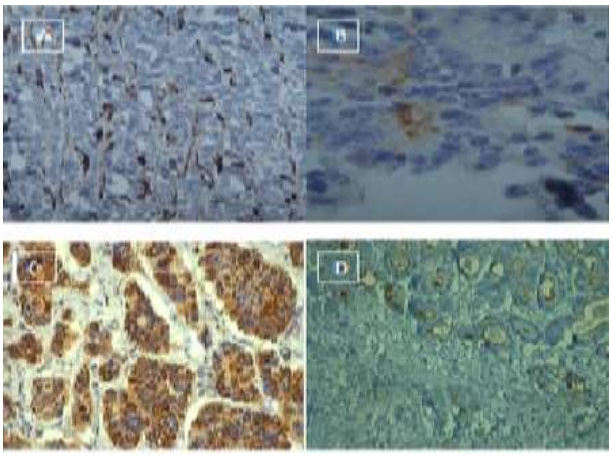

Figure 1. Immunohistochemical staining for P-glycoprotein and multi-drug resistance associated protein 1 using JSB-1 and MRP-r1 monoclonal antibodies, respectively. A) Strong multi-drug resistance associated protein 1 staining in retinoblastoma tissue. B) Weekly P-glycoprotein staining in retinoblastoma tissue. C) Strong multi-drug resistance associated protein 1 cytoplasmic staining in colorectal adenocarcinoma tissue (x400). D) Strong apical P-glycoprotein staining in colorectal cancer adenocarcinoma tissue (x200).

Results

Clinical information

A total of 26 patients were enrolled in this study with a median age of three years (two months to six years). Formalin fixed paraffin-embedded tumor tissues were from 14 males and 12 females. There were 19 unilateral and seven bilateral retinoblastoma tumors. Tables 1 and 2 listed other clinico-pathological features of patients. The mean follow-up was 27.3 months.

Clinical outcomes

Any type of recurrence in the same eye before enucleation or in the contralateral eye was considered as recurrence. Ten out of 26 tumors were not subjected to preoperative chemotherapy (CRD^- Group, Table 1). They were followed up from six to 44 months during which one of them demonstrated recurrence in the other eye. Sixteen patients received preoperative chemotherapy (CRD^+ Group, Table 2). The follow-up time was between 17 to 60 months; at this time, in six out of 16 cases recurrences were developed. The results showed the higher frequency of recurrence in CRD^+ group compared to CRD^- group.

Protein expression

MRP1 was expressed in three out of 10 tumors (30%) of patients in CRD^- group, while it was positive in nine out of 16 tumors (56.3%) in CRD^+ group. MDR1 was positive only in one tumor of patients (10%) in CRD^- group, whereas it was expressed in five out of 16 tumors (31.3%) in CDR^+ group. The results demonstrated the high frequency of expression for both proteins in CRD^+.

Protein expression and their correlation with clinico-pathological features

Six (23%) and 12 (46%) tumors were P-gp and MRP1 positive, respectively. MRP1 positive was observed in eight unilateral and four bilateral tumors, while P-gp positives were detected in five unilateral and one bilateral tumors. There was no significant correlation between laterality of the tumors and both proteins expression ($p>0.05$). Twelve patients demonstrated choroidal or optic nerve invasion of which five and four expressed MRP1 and P-gp, respectively. Five tumors simultaneously presented both proteins of which two had invasion. In addition to, the results showed no correlation between both proteins expression and other clinicopathologocal features including; age, sex, tumor differentiation, stages.

Table 1. Retinoblastomas enucleated who were not subjected to preoperative chemotherapy (group1).

No	Age/Sex	Differentiation	Stage (group)	Affected eyes	MRP1	MDR1 (P-gp)	Recurrence/ Follow-up time (Months)
1	1 years/M	MD, no invasion	E	Left	-	-	No/36
2	2 years/ M	MD, no invasion	E	Left	+	+	No/18
3	2 years/F	PD, no invasion	E	Right	+	-	No/30
4	2 years/M	PD, no invasion	E	Left	-	-	No/34
5	1 years/M	MD, no invasion	E	Right	-	-	No/14
6	2 months/F	MD, no invasion	E	Right	-	-	No/14
7	3 years/F	PD, diffuse choroidal invasion, post-laminar optic nerve invasion	E	Left	-	-	No/6
8	4 years/F	PD, pre-laminar optic nerve invasion	E	Left	-	-	No/14
9	2 months/F	WD,choroidal Invasion	E	Both	-	-	Yes*/44
10	1 year/M	PD, no invasion	E	Left	+	-	No/ 9

*: Recurrence after enucleation in her left eye, MRP1: Multi-drug resistance associated protein 1, MDR1: Multi-drug resistance 1, MD: Mildly differentiated, PD: Poorly differentiated, WD: Well differentiated

Table 2. Characteristics of retinoblastoma patients with enucleation as the final treatment with their preoperative chemotherapy (group 2)

No	Age/Sex	Differentiation	Stage	Affected eyes	MRP1	MDR1 (P-gp)	chemotherapy	No response to CRD	Recurrence/ Follow-up time (Months)
11	6 years/F	WD, diffuse choroidal Invasion, optic nerve invasion	E	Right	+	+	6 cycle VEC	-	No*/48(child dead)
12	3 years/F	PD, diffuse choroidal Invasion	D	Right	-	+	6 cycle VEC	+	No/38
13	4 years/M	PD, no invasion	D	Right	+	+	8 cycle VEC	-	Yes/36
14	3 years/F	PD, pre-laminar optic nerve invasion.	D	Both	-	-	13 cycle VEC	-	No/29
15	3 years/M	PD, pre-laminar optic nerve invasion	D	Both	+	-	6 cycle VEC	-	No/27
16	3 years/M	PD, no invasion	E	Both	-	-	6 cycle VEC	-	No/35
17	3 years/M	PD, no invasion	C	Left	+	+	6 cycle VEC	-	Yes/36
18	4 years/M	PD, pre-laminar optic nerve invasion.	D	Right	-	-	6 cycle VEC	+(vit seeds)	No/23
19	3 years/M	PD, no invasion	D	Left	-	-	6 cycle VEC	-	Yes/30
20	3 years/M	WD, focal, choroidal Invasion	D	Left	+	-	6 cycle VEC	+	No/17
21	2 years/F	PD, diffuse choroidal Invasion, pre-laminar optic nerve invasion	Left eye (stage E) Right eye (stage D)	Both (eyes enucleated)	+	+	6 cycle VEC	-	Yes/60
22	6 years/F	PD, choroidal Invasion, post-laminar optic nerve invasion	D	Both (right eye enucleated)	+	-	6 cycle VEC	+	No/27
23	3 years/F	WD, no invasion	E	Both(right eye enucleated)	+	-	8 cycle VEC	+	No/25
24	4 years/M	WD, no invasion	E	Left	+	-	8 cycle VEC	-	Yes/18
25	5 years/M	MD, diffuse choroidal Invasion, pre-laminar optic nerve invasion	D	Right	-	-	6 cycle VEC	+	No/18
26	4 years/M	PD, no invasion	E	Left	-	-	8 cycle VEC	+	Yes/26

*: The patient had extraocular invasion after enucleation without tumoral involvement of the cut end of optic nerve, CRD: Chemoreduction, MRP1: Multi-drug resistance associated protein 1, MDR1: Multi-drug resistance 1, MD: Mildly differentiated, PD: Poorly differentiated, VEC: Vincrystine, etoposide, carboplatin, vit seeds: Vitreous seeds, WD: Well differentiated

Protein expression and their correlation with clinical outcomes

In the CRD^- patients, two tumors were MRP1 positive and one P-gp-positive. In this group, one of the patients developed recurrence in the non-enucleated eye. To clarify the correlation between positive expression and drug response, we analyzed the possible association of positive expression and the recurrence of the tumors in CDR^+ patients. There was no significant correlation between positive expression of two proteins and clinical outcomes ($p>0.05$). In addition, from five tumors, which expressed both evaluated proteins, three developed recurrences of those one of children deceased after 48 months of follow-up because of bony metastasis. In addition, P-gp and MRP1 expressions were not associated with the disease free survival rate of patients ($p>0.05$) in the follow-up period. The expression of one of the two proteins was associated with a *relative risk* of recurrence as 0.3 in all patients, 0 in CRD^- group and 0.5 in CRD^+ group had recurrence of the tumor. The co-expression of MRP1 and P-gp were associated with a relative risk of recurrence as 1.5 in all patients, 0 in CRD^- group and three in CRD^+ group included these cases. These results indicate that co-expression of both of these proteins was associated with three times more recurrence rate of the tumor.

Protein expression and their correlation with tumor invasion to ocular coat

Additionally, P-gp positive was observed in equal percentage (11.5%) of tumors with invasion and non-invasive cases. MRP1 was positive in 26% of tumors with no invasion and 19% of tumors with invasion. Thereby, there was no association of these proteins with invasion of the tumor to the ocular coat in our study ($p>0.05$).

Discussion

In this investigation there was a significant expression of both P-gp and MRP1 in the CRD^+ group compared with CRD^- group. We found that there is a correlation between co-expression of two proteins and the relapse of tumor in retinoblastoma patients.

Chemotherapy has been known to be a therapeutic option in both cure and preservation of vision of retinoblastoma child. Hence, the prediction of clinical outcomes is considered to be the critical subject in this disease. A number of human ABC transporters have been contributed to multidrug resistances (MDR) in cancer treatment when over-expressed in cancerous cells. Expression of ABC transporters, such as P-gp and MRP1, have been detected in unilateral sporadic retinoblastomas.[17] P-gp expression can confer the drug resistance in retinoblastoma tumors, hence, the use of cyclosporin A in chemotherapy improved the clinical outcomes in children.[15] MRP1 was detected in patients who received triple chemotherapy beside cyclosporine, however, they were finally enucleated.[16] It seems that MRP1 protein enable to confer the different types of drug resistance in these tumors. The prognostic role of these proteins remained to be clarified. In this study, we investigated P-gp and MRP1 intrinsical expression in retinoblastoma tumors, and we evaluated their expression and their correlation to treatment response and any type of recurrences.

The results demonstrated that six (23%) and 12 (46%) tumors were P-gp and MRP1 positive, respectively. Our findings were in agreement with previous study of Wilson et al, 2006, that reported the number of tumors with MRP1 positive is more than P-gp positive.[17] Additionally; there was no association of these proteins with invasiveness of the tumor in our study. In addition, there was no association between these proteins and differentiation, laterality and stage of the tumors as pervious reports.[18,19]

Our study shows that expression of P-gp or MRP1 alone did not seem to be a predictive marker for the biological behavior of the tumor, however, the expression of both proteins in tumor might cause worse outcomes in children. Our finding is consistent with previous studies[18,19] that reported no correlation between MDR1/P-gp expression and clinical outcomes. However, the relative risk of recurrence was three times higher among children who co-expressed two proteins compared than those who expressed only one protein in all cases. But in the CRD^+ group this relative risk is much higher and appears to be six times more than CRD^- group. This results suggest that co-expression of both proteins might be an important factor

in recurrence of RB patients specially in the group with previous chemotherapy. The results show that chemotherapy agents could probably induce these proteins expressions and induction of both proteins expression could induce the tendency for secondary recurrent tumors from primary indolent retinoblastoma cells or de novo appearance of tumors from primarily normal appearing cells.

Our results indicated that the tumorigenesis role of these markers is likely independent of their putative role in drug resistance as previous study indicated[20] or facilitating the way for appearance of new tumors. Furthermore, our results did not indicate the significant correlation between disease free survival and the two protein expressions.

Conclusion

For the first time, we demonstrated the expression of P-gp and MRP1 proteins in some enucleated eyes from Iranian retinoblastoma tumors. We did not find the significant association between MRP1 and P-gp expression and clinical outcomes, however, recurrence was observed in a few patients who simultaneously presented both proteins. Thereby, further investigations with relatively large numbers of patients need to find out precisely the association of MRP1 and P-gp expression with RB disease.

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