• Basic Research •

Expression of multidrug-resistance associated proteins in human retinoblastoma treated by primary enucleation

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Received: 2017-11-17 Accepted: 2018-05-09

Abstract

• AIM: To reveal the expression of multidrug-resistance associated proteins: glutathione-S-transferase π (GSTπ), P-glycoprotein (P-gp) and vault protein lung resistance protein (LRP) in retinoblastoma (RB) without any conservative treatment before primary enucleation and to correlate this expression with histopathological tumor features.

• METHODS: A total of 42 specimens of RB undergone primary enucleation were selected for the research. Sections from the formalin-fixed, paraffin-embedded specimens were stained with HE and immunohistochemistry to detect the expression of GSTπ, P-gp and LRP.

• RESULTS: GSTπ was expressed in 39/42 (92.86%) RBs and in 9/9 (100%) well-differentiated RBs. P-gp/GSTπ was found in 30 (71.42%) of 42 RBs. Totally 9 (21.43%) tumors were well differentiated and 33 (78.57%) were poorly differentiated. Totally 15 (35.71%) eyes had optic nerve (ON) tumor invasion, 36 (85.71%) had choroidal tumor invasion, and 14 (33.33%) had simultaneous choroidal and ON invasion. There was no statistically significant relationship between P-gp, GSTπ, LRP positivity and the degree of ocular layer tumor invasion and ON tumor invasion (*P*>0.05).

• CONCLUSION: RB intrinsically expresses GSTπ, P-gp and LRP. GSTπ expression is positive in 100% well-differentiation ones, so in which way it is correlated with differentiation. But the other two proteins expressions are not related to tumor differentiation and to the degree of tumor invasion. GSTπ may be a new target of chemotherapy in RB.

• **KEYWORDS:** glutathione-S-transferase π; P-glycoprotein; vault protein lung resistance protein; retinoblastoma; multidrug-resistance proteins

DOI:10.18240/ijo.2018.09.06

Citation: Tang LJ, Zhou LJ, Zhang WX, Lin JX, Li YP, Yang HS, Zhang P. Expression of multidrug-resistance associated proteins in human retinoblastoma treated by primary enucleation. *Int J Ophthalmol* 2018;11(9):1463-1466

INTRODUCTION

Retinoblastoma (RB) is the most common malignant tumor in children. In order to protect vision and avoid enucleation, chemotherapy is currently considered to be an important treatment for RB. Chemotherapeutic can induce tumor regression and allow for focal treatments, such as cryotherapy and photocoagulation[1-4]. It is reported that fewer eyes were lost because of the use of ophthalmic artery chemosurgery and intravitreal melphalan in RB[5]. And intra-arterial chemotherapy (IAC) provides significantly superior control for solid tumor, subretinal seeds, and vitreous seeds[6]. Presently, vincristine, etoposide, and carboplatin (VEC) have been using in clinical of eyeball preserving conservative management in RB[7]. Nevertheless, drug resistance and relapses remain a major problem. Chemoresistance may be tumor inherent, or occur during administration of chemotherapy drugs. Multidrug resistance (MDR) has been associated with elevated expression of some particular proteins. P-glycoprotein (P-gp) was considered as one of the important MDR proteins. It was known to cause an increased efflux of cytotoxic drugs from the tumor cells and had the ability to mediate MDR[8-9]. Glutathione-S-transferase (GST), a class of phase II xenobiotic metabolism enzymes, playing an important role in cellular detoxification[10]. In addition, glutathione-S-transferase π (GSTπ) might also participate in oncogenesis, tumor progression, metabolism and drug resistance[11]. Lung resistance protein (LRP), the major component of complex ribonucleoprotein particles called "vaults," like P-gp, is believed to be link to the MDR[3]. In the recent decades, extensive researches have been done on MDR in RB, and several MDR associated proteins were involved. But the available data in regard to primary drug resistance is scanty and controversial. And the expression of GSTπ in RB has hardly any mentioned. From literatures and clinical we found chemotherapy drug resistance phenomenon was common in RB. So we speculated that RB might have different degrees of natural drug resistance genes expression. Therefore we detected the expression of GSTπ, P-gp and

LRP by immunohistochemistry to analyze natural resistance phenomenon in RB. These findings might have significant implications for the treatment of RB and discover the new target of chemotherapy in RB.

SUBJECTS AND METHODS

Patients and Tissues Institutional Review Board approval was obtained in accordance with the Declaration of Helsinki. Forty-two patients with a diagnosis of RB as the advanced intraocular disease were included in the study. After mydriasis, the fundus examination determined whether the cases were unilateral or bilateral RB. All patients were treated at Zhongshan Ophthalmic Center of Sun Yat-sen University from 2004 to 2007, and all had undergone primary enucleation. The clinical information was obtained from the medical records. For each patient, the histologic specimen was retrieved from the pathology archives.

Histopathology Retrospective analysis was performed on RB specimens obtained by primary enucleation. Sections were fixed in formalin, and the paraffin-embedded specimens were stained with hematoxylin and eosin (HE). According to the estimated percentage of Flexner-Wintersteiner rosettes in the available sections, tumor was classified as well-differentiated (WD) (Flexner-Wintersteiner rosettes more than 50%) or poorly differentiated (PD) (Flexner-Wintersteiner rosettes less than 50%). Other histopathological features, such as choroidal and optic nerve (ON) invasion were evaluated in both groups[12]. The degree of choroidal invasion was classified in five subgroups: I, no invasion; II, minimal invasion (tumor cells destroying Bruch's membrane without invading the choroid to any depth); III, massive invasion (any choroidal involvement that is not minimal); IV, intrascleral invasion; and V, extrascleral invasion. ON tumor invasion was classified in four subgroups: I, no invasion; II, prelaminar invasion; III, postlaminar invasion; and IV, invasion through the resection line and/or subarachnoid space[12].

Immunohistochemistry Four-micrometer-thick specimen sections were deparaffinized with xylene and rehydrated through graded ethanol washes. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 10min at room temperature. Slides were washed with phosphate-buffered saline (PBS) 3 times. The slides were soaked in citric acid buffer (pH 6.0), and was heated by microwave for 8min to expose the antigen. Nonspecific protein binding was blocked using 1% bovine serum albumin (BSA)/Tris-buffered saline (TBS, pH 7.6) solution for 30min. Next, sections were incubated with the mouse anti-human monoclonal antibody for GSTπ, P-gp and LRP (Dako, Denmark) at room temperature in a humidified chamber for 1h. Then, sections were washed and incubated with biotinylated rabbit anti-mouse antibody (Dako, Denmark) for 30min. Immunostaining visualization was developed with diaminobenzidine (DAB, Dako, Denmark) and counterstained with hematoxylin.

Immunoreactivity Scoring Two ophthalmic pathologists independently assessed the expression of GSTπ, P-gp and LRP by microscopy, and the final interpretation was based on agreeing assessments. GSTπ, P-gp and LRP expressions were graded semi quantitatively as negative (-, <10%), weakly positive (+, 10%-25%), positive (++, 25%-50%) and strong positive (+++, >50%) of viable tumor cells stained positive.

Statistical Analysis Statistical analysis was performed using Spearman correlation coefficient analysis and χ^2 test. P value of less than 0.05 was considered statistically significant. All statistical analyses were using statistical-analysis software (SPSS 16.0, USA).

RESULTS

The mean age of patients was 20mo, and the group was composed of 59.52% boys (n=25) and 40.48% girls (n=17). The tumor was unilateral in 35.71% of the cases (n=15) and bilateral in 64.29% (n=27). Histopathological assessment of the patients revealed 9 (21.43%) well differentiated, and 33 (78.57%) poorly differentiated. Fifteen (35.71%) eyes had ON tumor invasion, 36 (85.71%) had choroidal tumor invasion, and 14 (33.33%) had simultaneous choroid and ON invasion (Table 1). GSTπ expression was in 30 (92.86%) of 42 tumors, and the positive expression was related to the tumor differentiation (P<0.05). However, P-gp (78.57%) and LRP (33.33%) proteins expression showed no evident correlations to the differentiation of the tumor (P>0.05, respectively). Immunohistochemistry revealed granular cytoplasmic expression of GSTπ (Figure 1), granular cellular membrane or (and) cytoplasmic expression of P-gp, and cytoplasmic expression of LRP (Figure 2). LRP expressed in 14 (33.33%) of 42 tumors with a cytoplasmic pattern. the other two proteins showed no evident correlations to the differentiation of the tumor (P>0.05, respectively; Figure 3). There was no statistically significant relationship between the expressions of P-gp, LRP or GSTπ and choroidal invasion, ON invasion (P>0.05, respectively).

DISCUSSION

RB is the most common ocular malignant tumor in little children. In order to preserve vision and avoid enucleation, chemotherapy is currently an important modality for the treatment of RB. However, local tumor recurrence remains a major problem due to drug resistance. MDR has been linked to elevated expression of particular proteins, such as multidrug resistance proteins (MRP), LRP, breast cancer resistant protein (BCRP), and so on. Our results showed that drug resistance associated proteins of GSTπ, P-gp and LRP differently expressed in RB treated with primary enucleation. GSTπ was positive in most (92.86%) of 42 tumors. GST-π, the cytosolic detoxification protein, is generally in synergy with

Table 1 Summary of immunohistochemical findings

Patients	Differentiation	GSTπ	P-gp	LRP	Degree of invasion	
					Choroid	ON
1	WD	+++	+++	-	I	I
2	PD	++	+	-	II	I
3	PD	-	+	-	IV	I
4	PD	+	+++	-	V	II
5	PD	-	+++	-	V	I
6	PD	+++	++	-	III	III
7	WD	+++	+++	-	III	I
8	PD	+++	++	+	V	II
9	PD	++	+	+	II	I
10	PD	++	+++	+++	III	I
11	PD	++	+++	+	II	I
12	PD	+++	+++	-	I	I
13	WD	+++	+++	+	I	II
14	WD	+++	+++	-	I	I
15	PD	++	+	-	II	I
16	PD	++	+++	+	IV	IV
17	PD	+	+	-	IV	II
18	PD	++	++	+	V	III
19	PD	+++	+++	++	V	IV
20	PD	+++	++	+	IV	II
21	PD	+++	+++	-	II	II
22	PD	+	++	-	II	I
23	WD	+++	+	-	II	I
24	PD	+	++	-	IV	IV
25	WD	+++	+++	-	II	I
26	PD	+	++	-	V	I
27	PD	+	+	-	IV	I
28	WD	+++	-	++	II	I
29	PD	+++	-	-	III	I
30	PD	+++	-	-	I	I
31	PD	++	-	-	IV	II
32	PD	++	-	-	III	II
33	PD	+	-	-	I	I
34	PD	+	+	+	V	IV
35	WD	++	-	-	IV	I
36	PD	+	-	+	V	III
37	PD	+++	+++	-	III	I
38	PD	++	-	+	II	I
39	PD	-	++	++	III	I
40	PD	+++	+++	-	II	I
41	WD	+++	+++	-	II	I
42	PD	+++	+++	-	I	I
Positive (*n*)	/	39/42	33/42	14/42	35/42	15/42
Positive (%)	/	92.86	78.57	33.33	83.33	35.71

"+++", "++" and "+" are considered as positive staining, "–" are considered as negative staining; PD: Poorly differentiated; WD: Well differentiated.

multidrug resistance proteins-1 (MRP1) to confer resistance to the chemotherapeutic agents, especially platinum drugs[8,13-14]. Expression of the lung GST-π, LRP and MRP1 were all significantly upregulated in sphere-derived cells[15].

However, GSTπ has been scantly involved in RB drug resistance research so far. We found higher GSTπ expression rate in well differentiated tumors (100%) than in poor differentiated tumors (90.91%). This suggests that most untreated RB intrinsically express GSTπ. And drug resistance

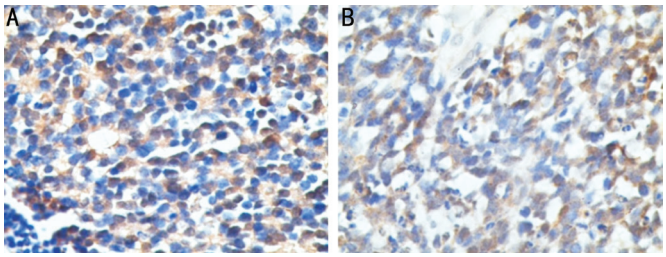

Figure 1 Photomicrograph showing GSTπ immunoreactivity in RB Positive staining in WD (A) and PD (B) tumor tissue. Microphotograph showing GSTπ in the cellular cytoplasm or (and) nucleus of the tumor cells (Magnification×400).

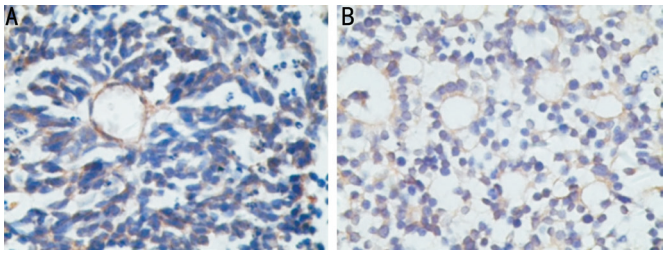

Figure 2 Photomicrograph showing P-gp immunoreactivity in RB Positive staining in PD (A) and WD (B) tumor tissue. Apical membrane staining of rosette formations. Microphotograph showing P-gp in the membrane of the tumor cells (Magnification×400).

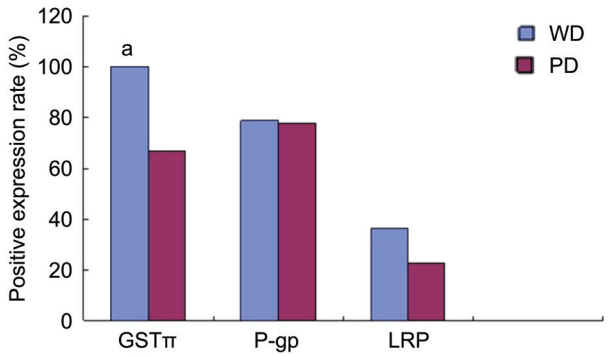

Figure 3 Compared of GSTπ, P-gp and LRP expressions in poorly differentiated and well differentiated RB [a]$P<0.05$, indicates the significant difference from WD RB.

more likely happens in differentiated RB. P-gp was vital in a series of RB cell lines and samples from RB patients who showed clinically resistance to chemotherapy[13]. We observed higher P-gp expression in WD tumors than PD tumors but without statistical significance. Insufficient case count might be the cause of the failure to show statistical significance. P-gp is more frequently expressed in RB enucleated after chemotherapy[12]. We found that P-gp is intrinsically expressed in 78.57% of our RB tumors. Our result was similar to other reports[16]. However, It was observed that P-gp was expressed only 26.7% in RB treated with primary enucleation[17]. There was no statistically significant difference between the two groups in regard to the degree of ocular choroidal invasion and ON invasion. However, 92.86% RB expressed GSTπ, which suggested that GSTπ might be a new target in chemotherapeutic treatment of RB.

Interestingly, we observed that the expression of LRP (33.33%), another important MDR associated protein[18], which was rarely expressed in our research. The result was obviously different from other researches. While, other researches considered that the overexpression of LRP could predict the sensitivity of cancer patients to chemotherapy and prognosis, and the expression of LRP was associated with primary resistance to vincristine, doxorubicin and platinum compounds[19]. We presumed that LRP might not work well as a resistance index in RB before chemotherapy, and it might only be some relationship with secondary drug resistance.

RB intrinsically expresses GSTπ, P-gp and LRP. The expression of the three resistance proteins may not be induced by chemotherapy, and there is nothing relationship with the degree of tumor invasion. The high expression of GSTπ, P-gp and LRP may lead to chemotherapy failure. And only GSTπ expression was related to tumor differentiation. The results could explain the chemotherapy drug resistance phenomenon of RB in clinical. In addition, GSTπ protein may be the novel and effective target of chemotherapy in RB. These findings might have important implications for the treatment of RB patients.

ACKNOWLEDGEMENTS

Foundation: Supported by the National Natural Science Foundation of China (No.30371515).

Conflicts of Interest: Tang LJ, None; **Zhou LJ,** None; **Zhang WX,** None; **Lin JX,** None; **Li YP,** None; **Yang HS,** None; **Zhang P,** None.

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