Immunohistochemical Detection of Multidrug-Resistant Protein Expression in Retinoblastoma Treated by Primary Enucleation

Matthew W. Wilson,1,2 Charles H. Fraga,3 Christine E. Fuller,4 Carlos Rodriguez-Galindo,5 John Mancini,5 Nikolaus Hagedorn,5 Markos L. Leggas,5 and Clinton F. Stewart5

PURPOSE. To compare the expression of multidrug-resistant proteins in retinoblastoma tumors among eyes treated with primary enucleation.

METHODS. A group of 18 patients with unilateral retinoblastoma with advanced intraocular disease was selected for the study. All patients had undergone primary enucleation. A histologic specimen from each patient was retrieved from the pathology archives and a tissue gene microarray was constructed (0.6 × 3–4 mm). Standard immunohistochemical techniques were used to study the tissue microarrays for the expression of the ATP-binding cassette (ABC) transporters: breast cancer resistance protein (BCRP; ABCG2), multidrug-resistant protein 1/P-glycoprotein (MDR1/Pgp; ABCB1), multidrug-resistant–associated protein 1 (MRP1; ABCC1), MRP2 (ABCC2), and MRP4 (ABCC4).

RESULTS. Of the 18 specimens retrieved, 16 had adequate tissue for study. MRP1 was expressed in 8 (50%) of 16 tumors, and MRP2 was expressed in 5 (31%) of 16 tumors. MDR1/Pgp was found in 2 (12%) of 16 retinoblastomas. MRP4 and BCRP were not detected in any of the tumors studied.

CONCLUSIONS. The results show that multiple ABC transporters were present in a cohort of sporadic patients with unilateral retinoblastoma who underwent primary enucleation. Studies are planned of the expression of ABC transporters in eyes treated by chemotherapy and/or radiation as a comparison with this group. (Invest Ophthal Vis Sci. 2006;47:1269–1273) DOI:10.1167/iovs.05-1321

Retinoblastoma is the most common primary intraocular malignancy of childhood.1 External beam radiotherapy (EBRT) was the standard of care for advanced bilateral disease until the 1990s. The increased incidence of secondary malignancies and the disfigurement caused by the ensuing orbital hypoplasia led investigators to seek alternative means of treatment.2 As a result, regimens incorporating systemic chemotherapy as a means to obtain early cytoreduction followed by treatment regimens for intraocular retinoblastoma.3–13 Different combinations of carboplatin, vincristine, etoposide or teniposide, and cyclosporine form the basis of most current treatment regimens for intraocular retinoblastoma.3–15

Although early reports on the treatment of intraocular retinoblastoma with chemotherapy plus focal therapy have yielded outcomes superior to those found with traditional EBRT alone, there are still tumors that prove resistant to the administered chemotherapeutic agents.14–16 Drug resistance can occur at multiple levels: host drug metabolism, drug delivery, microenvironment, and cellular mechanisms. Of these, only the cellular mechanisms of drug resistance have been studied in detail.

The ATP-binding cassette (ABC) transporters are membrane-bound proteins that efflux xenobiotics from the liver, kidney, and gastrointestinal tract and limit the penetration of these compounds to vital structures, such as the brain, placenta, and testis. In addition, they are thought to confer resistance by the energy-dependent efflux of chemotherapeutic agents from a cell. The functional protein comprises two nucleotide-binding folds and two transmembrane domains. Multidrug-resistant protein 1/P-glycoprotein (MDR1/Pgp) was the first human ABC transporter identified and was reported by Juliano and Ling.17 A 170kDa protein, MDR1/Pgp has been studied exhaustively for both its role in normal physiology and its possible role in drug resistance.18 MDR1/Pgp has been shown to be overexpressed in drug-resistant cells and to transport a variety of chemotherapeutic families, including the plant alkaloids, alkylating agents, anthracycline antibiotics, and antimetabolites. The most reproducible data to date involve leukemic cells. MDR1/Pgp is expressed in acute myelogenous leukemia in 30% of the patients at diagnosis, but in more than 50% of those patients who relapse.19–21 The effects of MDR1/Pgp have been shown to be mitigated by using either chemotherapeutic agents that are not potential substrates for transport or drugs such as verapamil and cyclosporine that are known inhibitors.22–24 This has resulted in improved efficacy of treatment.22

Chan et al.25,26 were the first to show the increased expression of MDR1/Pgp in retinoblastoma. Using immunohistochemistry, they showed that five multidrug-resistant cell lines produced higher levels of MDR1/Pgp than four chemosensitive counterparts. Further clinical studies documented improved clinical outcomes in patients with retinoblastoma in whom chemotherapy was supplemented with cyclosporine.7,26 In a later study, Chan et al.25 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.27

To date, 48 human ABC transporters have been identified, that have been classified into seven subfamilies, ABCA through -G.28 These transport proteins have been studied extensively for their ability to efflux anticancer drugs. Thus far, reports have shown that these drugs are substrates for 13 ABC transporters. The drugs most commonly used to treat retinoblastoma—carboplatin, vincristine, etoposide, and teniposide—are sub-

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strategies for some of the most commonly studied ABC transporters, including the breast cancer–resistant protein (BCRP; ABCG2), MRP2 (ABCC2), and MRP4 (ABCC4).\textsuperscript{29,30} Few data have been published regarding the expression of these transport proteins in retinoblastoma. Herein, we compared the expression of BCRP, MRP1, MRP2, MRP4, and MDR1/Pgp in retinoblastoma tumors among eyes treated with primary enucleation.

**METHODS**

Institutional Review Board approval was obtained in accordance with the Declaration of Helsinki. Eighteen patients with unilateral retinoblastoma with advanced intraocular disease were selected for the study. All patients were treated at St. Jude Children’s Research Hospital from January 1996 to April 2004, and all had undergone primary enucleation. Eyes were fixed in formalin and embedded in paraffin as pupil–optic nerve sections. For each patient, the histologic specimen was retrieved from the pathology archives, and a tissue microarray was constructed (0.6 × 3–4 mm). Two duplicate specimens from each tumor were placed on the array. Paraffin-embedded sections were stained with standard avidin biotin complex immunohistochemical techniques and streptavidin-horseradish peroxidase (SA-HRP), combined with an antigen retrieval step (Dako Corp., Carpinteria, CA) and the addition of an avidin-biotin (Vector Laboratories, Burlingame, CA) blocking step before application of primary antibodies. The following monoclonal antibodies against the specified ABC transporters were evaluated in the tissue microarray: MDR1/Pgp clone JSB-1 (Chemicon International, Temecula, CA), MRP1 clone MRPr1 (Kamiya, Seattle, WA), MRP2 clone M2III-6 (Kamiya), MRP4 clone M 4I-10 (Alexis Biochemicals/Axxora LLC, San Diego, CA), and BCRP clone BXP-21 (Kamiya). All antibodies were titrated for optimal immunoreactivity with minimal background (nonspecific) staining. Two of the antibodies were rat monoclonals, anti-MRP1 and anti-MRP4 used at 5 and 0.3 μg/mL, respectively. The three remaining antibodies were murine monoclonals: anti-MRP2, anti-MDR1/Pgp, and anti-BCRP used at 2, 4, and 0.25 μg/mL, respectively.

Primary antibodies were incubated overnight at 4°C followed by secondary antibody, either biotinylated anti-rat or biotinylated antimouse, at a dilution of 4 μg/mL. Results were visualized with diaminobenzidine (DAB; Dako Corp.), counterstained with hematoxylin (Dako Corp.), and mounted in permanent medium (Fisher Scientific, San Jose, CA).

### Table 1. Summary of ATP-Binding Cassette (ABC) Transporter Monoclonal Antibodies Tested and Used

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<th>Transporter</th>
<th>Monoclonal Antibodies Tested</th>
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<td>BCRP</td>
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### Table 2. Summary of Immunohistochemical Findings

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Positive (n) 0/16 8/16 5/16 0/16 2/16

*** Specimens were missing from the tissue microarray and excluded from the data set.
Suwannee, GA). Matched negative controls were incorporated that consisted of normal mouse IgG (Vector Laboratories) or rat IgG (BD-PharMingen, San Jose, CA) at the same concentration as the primary antibody used. Positive control specimens of paraffin-embedded human kidney sections were used for MDR1/Pgp, MRP1, MRP2, and MRP4. In addition, human tonsil specimens were included on the tissue microarrays as positive controls. Slides were reviewed independently by two pathologists (CEF, MWW), giving a total of four observations per tumor. Staining was scored as positive or negative in viable tumor; the intensity of staining was not quantified due to the inherent limitations of variability and subjectivity.

RESULTS

Multiple monoclonal antibodies were tested (Table 1). For purposes of this study, we selected those antibodies that showed minimal nonspecific staining. Our results are summarized in Table 2. Sixteen of the 18 tumors had adequate tissue for review. Only 12 (75%) of the 16 tumors expressed one or more of the ABC transporters studied. MRP1 expression was positive in 8 (50%) of 16 tumors, whereas MRP2 expression was observed in 5 (31%) of 16 and MDR1/Pgp in 2 (12%) of 16 (Figs. 1, 2, 3). All three proteins localized to cellular membranes and showed a greater intensity of staining in apical segments of observed rosettes. Two tumors expressed both MRP1 and -2, whereas one tumor expressed both MRP1 and MDR1/Pgp. MRP4 and BCRP staining were not observed within tumor cells; however, our respective controls of tonsil and vascular endothelium showed expected staining of cellular membranes (Figs. 4, 5).

DISCUSSION

Our results show variable expression of the ABC transport proteins in our cohort of 16 intraocular retinoblastomas treated with primary enucleation. We detected transporters in only 12 of the 16 tumors studied. MRP1 was most prevalent, with 50% of the tumors staining positive. MRP2 was present in 31% of tumors and MDR1/Pgp in 12%. MRP4 and BCRP immunoreactivity were not observed in viable tumors in any of the specimens studied. Three tumors expressed more than one ABC transporter: two showing MRP1 and -2 and one showing MRP1 and MDR1/Pgp. Based on our findings, we are left to conclude that the in vivo expression of major ABC transport proteins in untreated retinoblastoma is uncommon.

Chan et al. first described the multidrug-resistant phenotype in 5 of 11 chemonaı¨ve human retinoblastoma cell lines and, in a subsequent study, used immunohistochemistry to show high levels of MDR1/Pgp in five chemoresistant cell lines. A later study by Chan et al. found increased expression of MDR1/Pgp in 3 of 18 tumors treated with primary enucleation compared with three of three eyes enucleated after chemotherapy failed. Chemotherapy was believed to have selected resistant clones of cells expressing MDR1/Pgp. Their data provided the basis for the recommendation of incorporating cyclosporine, an inhibitor of MDR1/Pgp, into existing chemotherapy regimens of carboplatin, vincristine, and etoposide and contributed to their observed improvement in clinical outcomes. When tumor recurrences ensued, even with the use of cyclosporine, MRP1 was put forth as one possible explanation. Increased levels of MRP1 were found in eyes treated with carboplatin, vincristine, etoposide, and cyclosporine that were eventually enucleated. MRP1 may have provided alternative means of inducing drug resistance.

More recently, Krishnakumar et al. observed MDR1/Pgp in 23 (38%) of 60 intraocular tumors treated with primary
enucleation. They correlated MDR1/Pgp expression with clinical outcome. In three patients who received adjuvant chemotherapy based on high-risk histologic features, metastases developed, despite their having primary tumors that were negative for MDR1/Pgp. There was no correlation between MDR1/Pgp expression and clinical outcome. Patients whose tumors did not express MDR1/Pgp appeared to have a worse outcome.

Our results, when compared with those of Chan et al. and Krishnakumar et al., show that there was no uniformity of MDR1/Pgp expression in untreated retinoblastoma. These differences may be attributed to the monoclonal antibodies used. We used only those that localized to the apical cell membranes in our control tissue and minimized nonspecific staining. In contrast to other studies, we used a microarray technique, studying representative samples from each tumor. Critics may argue that the microarray technique does not permit adequate evaluation of the entire tumor. However, we believe that the microarray technique perpetuates the fundamental pathology principle of representative tumor sampling. Unless an entire tumor is sampled, no conclusive statements can be made. Previous reports using microarrays to study the ABC transporters substantiate our technique.32

The variable expression of the ABC transporters, especially MDR1/Pgp, may explain why comparable results have been achieved by those researchers who have chosen not to use cyclosporine in the management of retinoblastoma. Increased drug accumulation and drug-resistance reversal with MDR1/Pgp inhibitors have been well documented in vitro, but only suggested in clinical trials. The failure to substantiate the clinical relevance of MDR1/Pgp and its inhibitors can be attributed to the absence of confirmation of MDR1/Pgp expression in treated tumors, a lack of evidence of MDR1/Pgp inhibition in vivo, and MDR1/Pgp inhibitor toxicity at doses necessary to achieve serum concentrations comparable to those that were effective in laboratory models. Of these, it is the failure to substantiate any uniform expression of MDR1/Pgp in untreated retinoblastoma that is the most notable.

Because we limited our study to untreated intraocular tumors, we cannot comment on the possibility of the induced expression of MDR1/Pgp, MRP1, MRP2, MRP4, and BCRP by chemotherapy or the selection of tumor cells expressing these proteins de novo. Further study of eyes enucleated after exposure to chemotherapy is needed. As for the expression of MDR1/Pgp, MRP1, and MRP2, it must be noted and evaluated in light of what it represents: a preliminary attempt to gain better understanding of the cellular mechanism of chemoresistance. Until proper clinical trials document improved outcomes by inhibition of ABC transporters in tumors shown to express these proteins, the importance of the expression of these proteins remains speculative. In the interim, attention should be paid to host metabolism, drug delivery, and microenvironment.

References

Multidrug-Resistant Protein Expression in Retinoblastoma


