Multidrug resistance gene expression in pediatric primitive neuroectodermal tumors of the central nervous system

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Pediatric primitive neuroectodermal tumor (PNET) is a malignancy of the central nervous system currently treated with surgery, radiation therapy, and chemotherapy. Despite aggressive management, tumors recur in almost one-half of all patients. Drug resistance of tumor cells may, in part, explain the poor outcome. Resistance to chemotherapeutic agents may be related to expression of the multidrug resistance gene (MDR1) and its protein product, P-glycoprotein.

The role of MDR1 in 16 instances of PNET was investigated using Western blot analysis to detect the expression of P-glycoprotein, messenger ribonucleic acid (mRNA), polymerase chain reaction to detect MDR1 mRNA expression, and Southern blot analysis to assess gene amplification. Analysis of proteins extracted from 13 tumors revealed that two of the 15 patients expressed detectable levels of P-glycoprotein. Polymerase chain reaction of ribonucleic acid from 12 PNET's revealed that six of the 12 patients (four of 10 de novo tumors and both recurrent tumors) expressed MDR1 mRNA. Southern blot analysis of deoxyribonucleic acid from 16 PNET's revealed no evidence of MDR1 amplification in any tumor. This is the first report of MDR1 expression in pediatric brain tumors. These data suggest a possible role for MDR1 in de novo and acquired drug resistance in PNET's.

KEY WORDS • multidrug resistance gene • brain neoplasm • pediatric tumor • primitive neuroectodermal tumor • polymerase chain reaction

PEDiATRIC primitive neuroectodermal tumors (PNET's) represent 20% of all primary neoplasms of the central nervous system (CNS) in children. Current treatment consists of surgery, radiation therapy, and, in high-risk patients with extensive tumors, chemotherapy.8 Not all patients benefit from chemotherapy, and over 40% have tumor recurrence within 5 years despite aggressive treatment, including adjuvant chemotherapy. The reasons for PNET recurrence following chemotherapy and the resistance to chemotherapy of recurrent tumors have not been elucidated.

Drug resistance remains a significant obstacle restricting the efficacy of chemotherapy for malignancies.10 Tumor cells may be unresponsive to chemotherapy at initial diagnosis (de novo drug resistance) or they may become refractory to treatment following cytotoxic therapy (acquired drug resistance). Expression of the multidrug resistance gene (MDR1) has been demonstrated to confer drug resistance to tumor cells in vitro.22,23 The gene encodes a 170-kD membrane-associated glycoprotein, P-glycoprotein, which functions as an energy-dependent drug efflux pump, decreasing intracellular cytotoxic drug concentrations to sublethal levels.13,20,24 The role of MDR1 and P-glycoprotein in pediatric CNS tumors has not been previously described.

We evaluated the expression of MDR1 in pediatric PNET by investigating tumor protein for the presence of P-glycoprotein, tumor ribonucleic acid (RNA) for the presence of MDR1 messenger RNA (mRNA), and tumor deoxyribonucleic acid (DNA) for MDR1 amplification.

Materials and Methods

Tumor Tissue Selection

A portion of all CNS tumor specimens removed at Childrens Hospital of Los Angeles between July, 1988,
and January, 1990, were immediately frozen in liquid nitrogen. Frozen specimens of pathologically verified PNET were ground in a cold (-70°C) mortar and pestle for extraction of protein, RNA, and DNA.

Western Blotting for Tumor Protein

Total protein was isolated using a previously described method.\(^2\) A 20-μL preparation of protein (60 μg) was combined with an equal volume of sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% vol/vol glycerol, 2% sodium dodecyl sulfate (SDS), 5% vol/vol 2-mercaptoethanol, and 0.001% bromophenol blue), and was separated electrophoretically on a 7.5% SDS-polyacrylamide gel with running buffer (25 mM Tris base, 62.5 mM glycine, and 0.1% SDS). Following electrophoresis, the gel was electroblotted to nitrocellulose* in transfer buffer (25 mM Tris base, 192 mM glycine, and 20% methanol). The nitrocellulose filter was stained for P-glycoprotein using an anti-P-glycoprotein monoclonal antibody (JSB-1) and a biotinylated goat anti-mouse antibody streptavidin alkaline phosphatase conjugate technique.\(^4\)\(^1\)

Extraction of RNA and Polymerase Chain Reaction

Total cellular RNA was isolated using an acid guanidinium isothiocyanate-phenol chloroform extraction method.\(^5\) The quality of tumor RNA was evaluated using agarose formaldehyde gel electrophoresis. For every sample, distinct 28S and 18S bands were seen, verifying the integrity of the RNA. The polymerase chain reaction (PCR) primers were oligonucleotides homologous to sequences specific to MDR1 mRNA: 5’-TTTTCATGTATAATGCAGAC-3’ (corresponding to bases 877 to 896) and 5’-TCCAAGAACAGGACTGATGG-3’ (corresponding to bases 1102 to 1083). The probe was an oligonucleotide sequence specific to the 226 base-pair PCR product: 5’-ATTACACGTGGTTGAAGCTAAC-3’ (corresponding to bases 1045 to 1068). The PCR primers for MDR1 were derived from exons one and two; thus, the 226 base-pair product is generated only from the reverse transcription of spliced mRNA. Contaminating genomic DNA generated a 767 base-pair product that was readily distinguishable from the PCR product derived from RNA transcriptions.

A concomitant β-actin mRNA PCR control was performed for every sample of tumor RNA. Previously described primer sequences were specific for the β-actin gene.\(^7\) A 50-μL reaction mixture contained 250 ng of RNA, 50 pmol each of 5’ and 3’ oligonucleotide primer; 2-deoxyadenosine triphosphate, 2-deoxyctytosine triphosphate, 2-deoxyguanosine-5’-triphosphate, and deoxythymidine triphosphate (1.25 mmol/liter); and reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 0.01% gelatin). The PCR’s were optimized for each primer pair; MDR1 reaction mixtures contained 1.0 mM MgCl\(_2\) and β-actin reaction mixtures contained 2.0 mM MgCl\(_2\) and a 1:50 dilution of “Perfect Match.”\(^\dagger\)

The RNA reaction mixture was first incubated at 94°C for 2 minutes. Reverse transcription was accomplished by means of 2.5 U reverse transcriptase (from avian myeloblastosis virus) and incubation for 75 minutes at 50°C. Primer extension was started by the addition of 1 U Taq polymerase;\(^8\) subsequently, denaturing, annealing, and extension steps were performed at 94°C for 1 minute, at 50°C for 2 minutes, and at 72°C for 3 minutes for 30 cycles in a DNA Thermal Cycler.\(\ddagger\) Contamination was minimized by preparing and storing PCR reagents and aliquots in areas free from PCR-amplified products and utilizing negative controls for each experiment. Then 10 μL of the PCR mixture was separated on a 2% agarose gel, the PCR product was transferred to nylon, and the membrane was dried and crosslinked with ultraviolet light. Southern blotting, hybridization with \(^32\)P end-labeled oligonucleotide probes, and washing were performed according to methods described previously.\(^17\) Autoradiograms were made with Kodak XAR film at room temperature for 20 to 60 minutes.

Southern Blotting of Tumor DNA

Total cellular DNA was extracted using a salting-out technique.\(^16\) Deoxyribonucleic acid samples were digested with the restriction enzyme EcoRI (a restriction endonuclease derived from Escherichia coli) and Southern blotting was performed by means of techniques previously described.\(^21\) The pMDR5α probe, HGM locus MDR1, was obtained from Dr. Michael Gottesman.\(^24\) The intensity of the autoradiographic bands was determined by densitometry,\(^2\) and used to estimate the copy number for amplified genes. The densitometry values for MDR1 of tumors expressing the gene were compared to those values of tumors that did not express MDR1 mRNA and a copy number ratio greater than 5 was taken to represent amplification.

Results

The clinical characteristics of the patients from whom the tumor specimens were derived are shown in Table 1. Samples of PNET’s from 15 patients were evaluated; tumors in 13 patients arose in the posterior fossa (medulloblastoma) and two patients had tumors of the cerebral hemisphere. The mean age of patients at diagnosis was 6 years (range 1 to 16.5 years). Twelve specimens were removed at initial diagnosis and four

\(\dagger\) Perfect Match manufactured by Stratagene, La Jolla, California.

\(\ddagger\) Taq polymerase manufactured by Ampli
taq, Cetus, Norwalk, Connecticut.

\(\ddagger\) Thermal Cycler manufactured by Perkin Elmer, Cetus, Norwalk, Connecticut.

* Densitometer, Model 620, manufactured by Bio-Rad, Richmond, California.
Drug resistance gene expression in neuroectodermal tumors

TABLE 1
Patient data in 15 instances of pediatric primitive neuroectodermal tumor*

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Sex</th>
<th>Age (yrs)</th>
<th>Tumor Location</th>
<th>Initial Tumor</th>
<th>Recurrent Tumor</th>
<th>P-Glycoprotein Expression</th>
<th>MDR1 mRNA Expression</th>
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*P-glycoprotein expression tested by Western blot analysis; multidrug resistance gene 1 (MDR1) messenger ribonucleic acid (mRNA) expression tested by RNA polymerase chain reaction. Abbreviations: pf = posterior fossa; po = parieto-occipital; fp = frontoparietal; = = negative test; + = positive test; 0 = not tested.

samples were of recurrent tumors. Both initial and recurrent tumor specimens were available from one patient.

P-Glycoprotein Expression

Western blot analysis of protein from 15 PNET's revealed expression of P-glycoprotein in two tumors (Fig. 1). Tumor tissue from Case 21 was obtained by resection at the second recurrence; previous therapy had consisted of two surgical resections, radiation therapy; and eight courses of a multidrug combination chemotherapy regimen which included vincristine. The other tumor revealing expression of P-glycoprotein was obtained from Case 9 at initial resection; the patient's parents refused any adjuvant therapy. The remaining 13 tumors demonstrated a faint 170-kD band that was also present in a normal brain control sample. As the intensity of these signals was significantly less than that in our positive samples and the signal was similar to that of normal brain, these specimens were termed "equivocal."

Expression of MDR1 mRNA

Analysis of the RNA PCR-amplified product from MDR1 mRNA revealed that six of 12 tumors expressed MDR1 mRNA. Four of 10 tumors resected at initial presentation and both recurrent tumors tested contained detectable MDR1 mRNA (Figs. 2A and 3A). Both of the tumor samples testing positive by Western blot analysis were also found positive by RNA PCR. The RNA extracted from normal nonmalignant hu-
human cerebral cortex was employed as a negative control and shown to be negative for MDR1 mRNA (Fig. 2A, Lane 1).

Of particular interest are two specimens obtained from Case 45, one at initial diagnosis and the other at recurrence. After this patient’s tumor was initially resected, she received eight weekly doses of vincristine concurrent with radiation therapy. She subsequently developed tumor progression necessitating a second tumor resection. The specimen obtained at recurrence, but not that from initial resection, demonstrated expression of MDR1 mRNA (Fig. 2A, Lanes 4 and 5).

**Specificity of MDR1 Primers for MDR1 mRNA**

To confirm the specificity of the primers for detecting MDR1 mRNA, PCR reactions were performed utilizing MDR1 primers and an MDR2 complementary DNA (cDNA) template. In no experiment was a 226-base-pair PCR product obtained.

**Gene Amplification of MDR1**

Southern blot analysis of tumor DNA digested with the restriction endonuclease EcoRI was performed using an MDR1 partial cDNA probe. All 16 tumors demonstrated five intact bands corresponding to molecular weights of 5.5, 3.35, 3.05, 1.7, and 0.82 kilobase pairs. Densitometric comparisons of DNA from tumors that expressed MDR1 mRNA versus those that did not show such expression revealed that no tumor displayed evidence of gene amplification.

**Discussion**

The data presented in this study suggest a possible role for MDR1 in both de novo and acquired drug resistance in pediatric PNET’s. Western blot analysis of protein from 15 PNET’s demonstrated that two tumors expressed increased amounts of P-glycoprotein; the remaining 13 tumors demonstrated a faint band at 170 kD. A similar band was seen in protein derived from normal human brain. The presence of this band was dependent on the JSB-1 anti-P-glycoprotein monoclonal antibody. This band may represent basal MDR1 expression derived from CNS capillary endothelium or cross-reaction with an unrelated endogenous protein. To further clarify Western blot results with regard to the equivocal 170-kD band we investigated the expression of MDR1 mRNA using PCR.

Results of PCR analysis revealed that six of 12 PNET’s expressed MDR1 mRNA; this included four of 10 primary tumors and both recurrent specimens tested. Tumor from the initial resection and from a recurrence following chemotherapy were available from Case 45. This patient’s recurrent tumor exhibited MDR1 mRNA expression, but the primary specimen did not. Four of the six tumors that tested positive by PCR were found negative by Western blot analysis. We believe that this represents the increased sensitivity of the PCR technique. Normal human brain was employed as a negative control. The PCR assay was adjusted so that the MDR1 expression by capillary endothelium in normal brain was below the limit of detection. Thus, the expression detected in the tumor samples is greater than that seen in normal brain. Polymerase chain reaction has been used as a quantitative assay for MDR1 expression by other investigators, and low levels of expression have been demonstrated to confer multidrug resistance.19

One disadvantage of the PCR technique is that the cells expressing the MDR1 cannot be identified. Thus, the increased expression seen in our tumor samples could be the result of an increase in the level of expression normally present in brain capillary endothelium or the result of induction of the gene in the tumor cells themselves. In either case, the result of increased MDR1 expression is a decrease in the intratumoral drug concentration.

Southern blot analysis of DNA from 16 PNET’s revealed no MDR1 amplification. This suggests that, as in other human malignancies, MDR1 amplification does not play a role in the development of in vivo drug resistance.

Matsumoto, et al.,15 have reported on the presence of P-glycoprotein in the membrane of human glial tumors using an immunohistochemical technique on frozen sections of surgical specimens. All 18 tumors examined contained cells expressing P-glycoprotein; the percentage of expressing cells ranged from 6.5% to 27.6%. Interestingly, the vascular endothelial cells in the tumors did not stain positive for P-glycoprotein. In normal brain, the vascular endothelium has been reported to express P-glycoprotein.24 This suggests that our results represent tumor MDR1 expression rather than false-positive CNS capillary endothelium sampling.

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*† cDNA template supplied by National Institutes of Health Repository of Human and Mouse DNA Probes and Libraries, American Type Culture Collection, Rockville, Maryland.*
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To date, there have been few clinical studies correlating the presence or amount of MDR1 expression with response to chemotherapy. A recent investigation of 30 pediatric patients with soft-tissue sarcomas, using immunohistochemical techniques to detect P-glycoprotein, demonstrated that MDR1 expression may be an important adverse prognostic variable. Nine of 30 patients expressed P-glycoprotein at some point in their therapy, and this group of patients had a significantly poorer complete response rate, as well as poorer relapse-free and overall survival rates, compared to patients who had no detectable P-glycoprotein. A study of pediatric patients with neuroblastoma found that significantly more tumors from patients treated with chemotherapy expressed MDR1 mRNA compared to samples from untreated patients, and that high-level expression was more common in patients following chemotherapy. However, no correlation between the amount of expression and the clinical response could be determined. Correlation between MDR1 expression and clinical outcome in a large population of patients will be necessary to determine the significance of MDR1 in pediatric PNETs.

P-glycoprotein confers resistance to some drugs, such as the antimitic agents and epipodophyllotoxins, which are increasingly used in therapy of pediatric brain tumors. In this regard, the two recurrent PNET specimens that tested positive are of interest. Paired specimens obtained at diagnosis and recurrence were available from Case 45; the recurrent tumor from this patient, but not her de novo specimen, expressed high levels of MDR1 mRNA. Intercurrent therapy consisted of irradiation and vincristine, a cytotoxic drug known to participate in the chemotherapy cross-resistance pattern of MDR1. Tumor tissue from Case 21, resected at a second recurrence, also expressed high levels of MDR1 mRNA. Previous treatment in this patient also included vincristine, among other chemotherapeutic agents.

Conclusions

In vitro studies have shown that MDR1 expression can be induced by growing drug-sensitive cells in the presence of sublethal concentrations of chemotherapeutic agents. Therapeutic strategies, aimed at overcoming MDR1 drug resistance have been employed in treatment strategies for non-CNS tumors. Similar strategies may play an important role in the treatment of pediatric PNET.

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