

## EXPRESSION OF THE GENE FOR MULTIDRUG-RESISTANCE-ASSOCIATED PROTEIN AND OUTCOME IN PATIENTS WITH NEUROBLASTOMA

MURRAY D. NORRIS, PH.D., SHARON B. BORDOW, B.SC., GLENN M. MARSHALL, M.B., B.S.,  
PAUL S. HABER, M.B., B.S., M.D., SUSAN L. COHN, M.D., AND MICHELLE HABER, PH.D.

**Abstract Background.** Overexpression of the gene for the multidrug-resistance-associated protein (*MRP*) has been linked with resistance to chemotherapeutic agents (multidrug resistance) in vitro. The expression of *MRP* by neuroblastoma cells correlates with *N-myc* oncogene amplification, a well-established prognostic indicator in patients with neuroblastoma.

**Methods.** To relate *MRP* gene expression to established prognostic markers and the clinical outcome of neuroblastoma, we analyzed *MRP* expression in specimens of primary tumors from 60 patients with neuroblastoma.

**Results.** Levels of *MRP* gene expression were significantly higher in tumors with *N-myc* amplification than in tumors without such amplification ( $P < 0.001$ ). High levels of *MRP* expression were strongly associated with reductions in both survival and event-free survival ( $P < 0.001$ ) in the overall study population and in subgroups of patients without *N-myc* amplification and patients with localized disease. For the overall study population, the five-

year cumulative survival rates in the groups with high and low levels of *MRP* expression were 57 percent (95 percent confidence interval, 37 to 78 percent) and 94 percent (95 percent confidence interval, 86 to 100 percent), respectively. In contrast, expression of the *MDR1* multidrug-resistance gene was not predictive of survival or event-free survival. After adjustment by multivariate analysis for the effects of *N-myc* amplification and other prognostic indicators, high levels of *MRP* expression retained significant prognostic value for poor survival (relative hazard, 14.9;  $P = 0.01$ ) and poor event-free survival (relative hazard, 9.7;  $P = 0.004$ ), whereas *N-myc* amplification had no prognostic value.

**Conclusions.** High levels of *MRP* gene expression in patients with neuroblastoma correlate strongly with poor outcome. The findings suggest that expression of this multidrug-resistance gene accounts for the association between *N-myc* amplification and reduced survival. (*N Engl J Med* 1996;334:231-8.)

©1996, Massachusetts Medical Society.

NEUROBLASTOMA is the most common solid tumor of early childhood.<sup>1</sup> Patients with localized disease have a favorable prognosis, but the majority of children with neuroblastoma present with metastases and have poor prognoses despite intensive multimodal therapy.<sup>2</sup> Treatment failure in these patients is largely attributable to resistance to a diverse range of structurally and functionally unrelated cytotoxic drugs. Resistance to multiple chemotherapeutic agents (multidrug resistance) is particularly apparent in patients whose tumors show amplification of the *N-myc* oncogene, one of the most powerful indicators of poor outcome in neuroblastoma.<sup>3</sup> The mechanisms by which such amplification influences the phenotype of neuroblastoma are unclear.

Multidrug resistance has been intensively investigated in the laboratory. Among the underlying mechanisms, the best-known involves the *MDR1* gene, which encodes P-glycoprotein.<sup>4</sup> Although a number of studies have suggested a role for *MDR1* in the chemoresistance associated with certain cancers,<sup>4</sup> the contribution of this gene to the multidrug-resistant phenotype of neu-

roblastoma is controversial.<sup>5-10</sup> Recently, another gene, the gene for multidrug-resistance-associated protein (*MRP*), has also been found to confer a multidrug-resistant phenotype in vitro.<sup>11-13</sup> The *MRP* gene, located on chromosome 16p13.1,<sup>14</sup> encodes a 190-kd membrane-bound glycoprotein that, like P-glycoprotein, mediates resistance to a range of drugs made from natural products, including the vinca alkaloids, anthracyclines, and epipodophyllotoxins.<sup>12,15</sup> We recently reported increased *MRP* expression in neuroblastomas with amplification of the *N-myc* oncogene and decreased *MRP* expression after the differentiation of neuroblastoma cells in vitro.<sup>16</sup> In the present study, we examined the relation between *MRP* expression and clinical outcome in patients with neuroblastoma. We found a significant association between high levels of *MRP* expression and poor outcome and showed that this relation is independent of *N-myc* amplification.

### METHODS

#### Patients and Tumor Specimens

Samples of 60 primary neuroblastoma tumors from untreated patients were obtained from either the Neuroblastoma Tumor Bank of the Pediatric Oncology Group or the Prince of Wales Children's Hospital in Sydney, Australia. The samples from the United States were sent to the investigators for analysis after the proposed study had been reviewed and approved by the Neuroblastoma Biology Subcommittee of the Pediatric Oncology Group. Because insufficient information was available for all patients to be classified uniformly according to the staging criteria of the International Neuroblastoma Staging System,<sup>17</sup> the Pediatric Oncology Group tumors were classified according to the staging system used by that group, which was based on the results of resection of the neuroblastoma,<sup>18</sup> and the tumors from the hospital were classified according to the system of Evans et al., which was based on the anatomical site of the tumor.<sup>19</sup> Among the 28 Pediatric

From the Children's Leukaemia and Cancer Research Centre, University of New South Wales and Prince of Wales Children's Hospital, Sydney, Australia (M.D.N., S.B.B., G.M.M., M.H.); the Department of Gastroenterology, Prince of Wales Hospital, Sydney, Australia (P.S.H.); and the Department of Pediatrics, Northwestern University Medical School and Children's Memorial Hospital, Chicago (S.L.C.). Address reprint requests to Dr. Michelle Haber at the Children's Leukaemia and Cancer Research Centre, Prince of Wales Children's Hospital, High St., Randwick, NSW 2031, Australia.

Supported by grants (to Dr. Norris, Dr. M. Haber, and Dr. Marshall) from the National Health and Medical Research Council (Australia) and the New South Wales State Cancer Council (Australia), by a grant from the Leo and Jenny Leukaemia and Cancer Foundation of Australia (to Dr. Marshall), and by the Children's Leukaemia and Cancer Foundation (Australia). Ms. Bordow is the recipient of an Australian Postgraduate Research Award.

Oncology Group tumors, there were 3 in stage A, 4 in stage B, 12 in stage C, 7 in stage D, and 2 in stage DS; the 32 tumors from the hospital comprised 8 in stage I (including 5 ganglioneuroblastomas), 5 in stage II, 8 in stage III, 9 in stage IV, and 2 in stage IVS. The clinical stages of the tumors were then classified as favorable (Evans stages I, II, and IVS and Pediatric Oncology Group stages A, B, and DS) or unfavorable (Evans stages III and IV and Pediatric Oncology Group stages C and D). Before the study, the number of copies of the *N-myc* oncogene per haploid genome were determined independently in each tumor by Southern blot analysis, with quantitation of the extent of amplification by serial dilution of DNA.<sup>20,21</sup> A tumor with more than 3 copies of *N-myc* was considered to have *N-myc* amplification; 13 of the tumors studied (7 in stages III or C and 6 in stages IV or D) had *N-myc* amplification, with from 8 to 200 copies. Data on histologic classification according to the system of Shimada et al.<sup>22</sup> were available for less than half the tumors; therefore, this factor was not analyzed.

All the patients received their diagnoses from December 1984 through September 1994 and were treated in a manner specific to their tumor stage. In the group from the hospital, patients in stages I, II, and IVS were treated with surgical resection of the primary tumor alone or with surgery and six months of chemotherapy with vincristine and cyclophosphamide; patients in stages III and IV received three months of chemotherapy (with teniposide, doxorubicin, cisplatin, vincristine, and cyclophosphamide),<sup>23</sup> delayed resection of the primary tumor, and radiation therapy at the primary site. Patients in stage III whose tumors had *N-myc* amplification and patients in stage IV subsequently received supralesional chemoradiotherapy and autologous bone marrow transplantation, as described elsewhere,<sup>24</sup> whereas all other patients in stage III completed 12 months of chemotherapy with the same agents that were used preoperatively.

Among the patients from the Pediatric Oncology Group, those in stage A were treated with surgery alone. Before 1987, all infants under one year of age who had unresectable tumors (those in stages B, C, and D) and all children one year of age or older with stage B disease were treated with five cycles of cyclophosphamide and doxorubicin. Infants in stage DS were also treated in this way or observed without treatment. Among infants with disease diagnosed after 1987 and unresectable tumors, those with hyperdiploid tumors were treated with cyclophosphamide and doxorubicin, whereas those with diploid tumors received cisplatin and teniposide. Older children with stage C disease received cyclophosphamide-doxorubicin alone or combined with cisplatin-teniposide, and in some cases also with radiation. Children with stage D disease were treated with the same pairs of drugs, given in alternation or together in pulses.<sup>25-30</sup>

Overall, eight patients underwent autologous bone marrow transplantation as part of their therapy. The responses of all the patients were assessed according to internationally accepted criteria,<sup>17</sup> and outcomes were determined as of June 1995. The median period of follow-up for the surviving patients was 33 months (range, 7 to 120), whereas the median time from diagnosis to death among the patients with treatment failure was 9 months (range, 2 to 23). The outcome measures studied were survival, defined as the time from diagnosis to death, and event-free survival, defined as the time from diagnosis to the first major event (relapse, failure to enter remission, or death). Among the patients who survived and those who survived without events, survival and event-free survival, respectively, were defined as the time from diagnosis to the last follow-up and were treated as censored observations. Although the Australian and American institutions differed in their treatment protocols, there was no significant difference between the two groups of patients in overall survival or event-free survival. Moreover, multivariate analysis indicated that the inclusion of the treatment center as a variable had no prognostic value.

#### Analysis of Gene Expression by the Polymerase Chain Reaction

The competitive assay of RNA by the polymerase chain reaction (RNA PCR assay) that we used is a well-established method of analyzing gene expression in a range of tumor tissues, including neuroblastoma.<sup>16,31-34</sup> The total amount of RNA in cytoplasm was isolated from frozen tumor tissue,<sup>35</sup> and complementary DNA (cDNA) was

synthesized from 1- $\mu$ g aliquots of RNA with random hexanucleotide primers and Moloney murine leukemia virus reverse transcriptase.<sup>31</sup> Aliquots of cDNA corresponding to 50 ng of RNA were amplified for 30 cycles in a final volume of 25  $\mu$ l with 1 unit of *Taq* polymerase. After an initial period of denaturation for 3 minutes at 94°C, the cycling conditions consisted of 45 seconds at 94°C, 45 seconds at 55°C, and 90 seconds at 72°C. Each target gene sequence (*MRP*, *MDR1*, or *TRK*) was amplified with a control gene sequence ( $\beta_2$ -microglobulin) with gene-specific oligonucleotide primers, as described elsewhere.<sup>16,31,33</sup> After electrophoresis of the PCR products on 12 percent polyacrylamide gel and staining with ethidium bromide, the bands were visualized and photographed under ultraviolet transillumination. Densitometric analysis was performed with photographic negatives,<sup>16,33,36</sup> and the ratio between the expression of the target gene and that of the control gene was determined for each sample by dividing the densitometrically determined volume of the target electrophoretic band by that of the control band. The level of expression of a given gene in an individual tumor (the PCR ratio) was defined as the average of the ratios for that gene obtained in competitive RNA PCR analyses performed on at least three occasions. All the PCR analyses were performed with the investigators unaware of the patients' survival status and outcome.

#### Statistical Analysis

Differences between groups of tumor specimens in the PCR ratios for specific target genes were assessed by two-sided Student's t-tests. In the survival analyses, the PCR ratios for specific genes in each tumor were categorized as low or high, according to the following procedures. For the *TRK* gene, tumor specimens were classified as having low expression if a *TRK*-specific PCR product was either absent or barely detectable (PCR ratio, <0.1) and as having high expression if the PCR ratio was  $\geq$ 0.1 (range among the specimens studied that had high expression, 0.13 to 1.68). For the *MRP* and *MDR1* genes, there was no clear demarcation between tumors with low expression and those with high expression; therefore, we used a different method of classification. Gene expression in an individual tumor was considered high if the PCR ratio for the gene in question exceeded the mean ratio for all 60 specimens. The decision to dichotomize the values obtained for *MRP* and *MDR1* expression around the mean PCR ratios was made a priori, not after examination of the results of the survival analysis.

The survival analysis was performed according to the method of Kaplan and Meier, and outcome was compared between subgroups by two-tailed log-rank tests for univariate comparisons. Associations between the patients' clinical characteristics and the molecular characteristics of the tumor specimens were examined by Fisher's exact test. A Cox proportional-hazards regression model was used in the multivariate analysis. Statistical analyses were performed with StatView 4.1 (Abacus Concepts, Berkeley, Calif.) or SAS software (version 6.08, SAS Institute, Cary, N.C.). Results are expressed as means  $\pm$ SE, and probabilities of survival and relative hazards are given with 95 percent confidence intervals.

## RESULTS

### Clinical and Molecular Characteristics

To determine whether the 60 patients whose tumors we studied were representative of patients with neuroblastoma in general, we analyzed survival in relation to four well-established prognostic signs (Fig. 1 and Table 1). The results were consistent with previous studies.<sup>20,37,38</sup> Amplification of the *N-myc* oncogene was seen in 22 percent of patients, all of whom had advanced-stage disease at diagnosis, and *N-myc* amplification was associated with significantly reduced survival ( $P=0.002$ ) (Fig. 1A). High levels of expression of the *TRK* proto-oncogene, which encodes a receptor for nerve growth factor, were present in 80 percent of patients and were

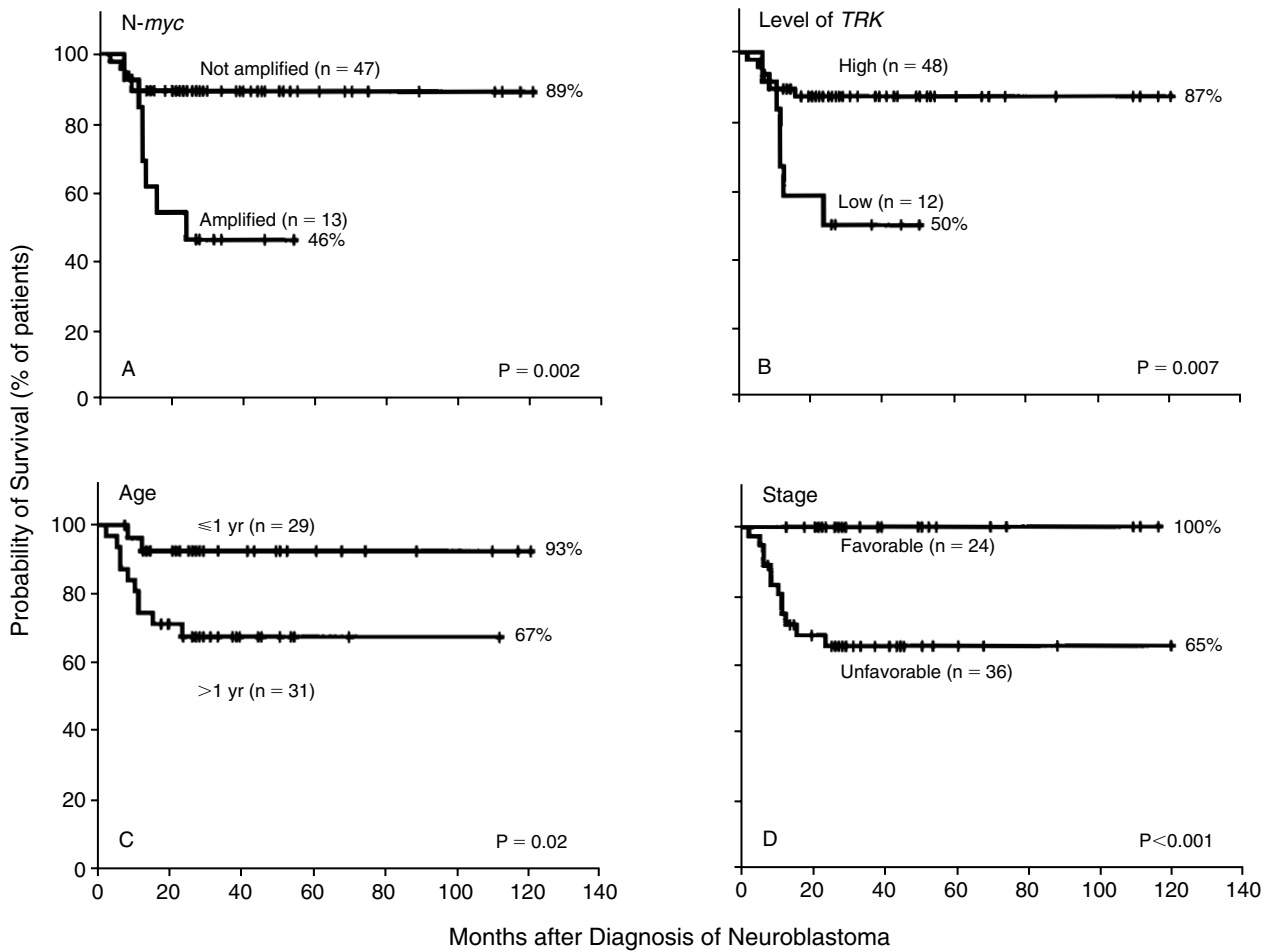


Figure 1. Cumulative Survival of 60 Patients with Neuroblastoma.

The Kaplan–Meier curves show the probability of survival with respect to the number of copies of *N-myc* found by Southern blot analysis (Panel A); the level of *TRK* expression, categorized as high or low according to the presence or absence of a clearly detectable *TRK* PCR product (Panel B); the patient's age at diagnosis ( $\leq 1$  or  $> 1$  year) (Panel C); and the tumor stage, categorized as favorable (Evans stages I, II, and IVS; Pediatric Oncology Group stages A, B, and DS) or unfavorable (Evans stages III and IV; Pediatric Oncology Group stages C and D) (Panel D). P values were determined by the log-rank test. Tick marks indicate the length of follow-up of individual patients who survived. The median follow-up after diagnosis among the surviving patients was 33 months (range, 7 to 120).

strongly associated with improved survival ( $P=0.007$ ) (Fig. 1B), as reported elsewhere.<sup>37</sup> *TRK* gene expression in tumors with amplification of the *N-myc* oncogene was significantly lower (median PCR ratio, 0.0) than in tumors without *N-myc* amplification (median ratio, 0.681;  $P=0.001$ ). Infants, 90 percent of whom (26 of 29 patients) expressed high levels of the *TRK* gene, had significantly better survival than older children ( $P=0.02$ ) (Fig. 1C). Tumor stage was a powerful prognostic indicator ( $P<0.001$ ) (Fig. 1D), with no deaths among patients with favorable tumor stages. The proportion of patients with unfavorable tumor stages (60 percent) was similar to that in other large series of patients with neuroblastoma.<sup>20,38,39</sup> However, in this study there was a smaller proportion (27 percent) of patients with stage IV or D tumors than would have been expected with consecutive enrollment,<sup>20,38,39</sup> because of the frequent diagnosis of stage IV or D disease by analysis of bone

marrow and urinary catecholamines, without biopsy of the primary tumor. When we studied event-free survival, *N-myc* amplification, *TRK* expression, and tumor stage continued to have significant prognostic value (data not shown). The characteristics of our study population with respect to well-established prognostic indicators and outcome were thus representative of patients with neuroblastoma in general.

#### Expression of *MRP* and *MDR1*

We detected expression of the *MRP* gene in all 60 tumors obtained at diagnosis (mean [ $\pm$ SE] PCR ratio,  $0.403 \pm 0.034$ ). As in our earlier study,<sup>16</sup> levels of *MRP* expression in samples of neuroblastoma with *N-myc* amplification (mean PCR ratio,  $0.628 \pm 0.074$ ) were significantly higher than those in tumors without amplification (mean ratio,  $0.340 \pm 0.034$ ;  $P<0.001$ ). There was an intermediate level of *MRP* expression (PCR ratio,

Table 1. Relation of Age and Tumor Stage at Diagnosis to the Molecular Characteristics of the Tumors in the 60 Study Patients with Neuroblastoma.\*

CHARACTERISTIC	N-myc AMPLIFICATION		TRK EXPRESSION		MRP EXPRESSION	
	ABSENT (N = 47)	PRESENT (N = 13)	HIGH (N = 48)	LOW (N = 12)	LOW (N = 35)	HIGH (N = 25)
	no. of patients					
Age†						
≤1 yr	26	3	26	3	17	12
>1 yr	21	10	22	9	18	13
Tumor stage‡						
Favorable	24	0	23	1	19	5
Unfavorable	23	13	25	11	16	20

\*N-myc amplification was considered absent if there were three or fewer copies of N-myc per haploid genome and present if there were more than three copies. The level of TRK expression was considered high if the PCR ratio was 0.1 or above and as low if the ratio was below 0.1. The level of MRP expression was considered low or high in relation to the mean level calculated for all tumors analyzed.

†Age (categorized as ≤1 or >1 year at diagnosis) was not significantly associated with any of the three molecular characteristics studied, or with tumor stage ( $P > 0.05$  for all comparisons).

‡Tumor stage was categorized as favorable (Evans stages I, II, and IVS and Pediatric Oncology Group stages A, B, and DS) or unfavorable (Evans stages III and IV and Pediatric Oncology Group stages C and D). Tumor stage was significantly associated with N-myc amplification ( $P < 0.001$ ), TRK expression ( $P = 0.02$ ), and MRP expression ( $P = 0.009$ ).

0.534±0.022) in the human neuroblastoma cell line, SK-N-SH, which we used for reference. MRP was expressed at significantly higher levels in tumors with unfavorable clinical stages (mean PCR ratio, 0.483±0.047) than in tumors with favorable stages (mean ratio, 0.282±0.040;  $P = 0.004$ ), a finding that supported a trend noted earlier.<sup>16</sup> No significant differences in levels of MRP expression were found between tumors from children younger than one year at diagnosis and tumors from older children, or between tumors with high levels of TRK expression and those with low levels. The MDR1 gene was expressed in 56 of the tumors (93 percent; mean PCR ratio, 0.199±0.026). In contrast to MRP, MDR1 was expressed at lower levels in tumors with N-myc amplification (mean PCR ratio, 0.115±0.033) than in tumors without such amplification (mean ratio, 0.222±0.031), although the difference was not statistically significant ( $P = 0.09$ ).

### Gene Expression and Outcome

Figure 2 shows cumulative survival according to levels of expression of the MRP and MDR1 genes. High levels of MRP expression were strongly associated with reduced survival (Fig. 2A). For the overall study population, the five-year cumulative survival rates in the groups with high and low levels of MRP expression were 57 percent (95 percent confidence interval, 37 to 78 percent) and 94 percent (95 percent confidence interval, 86 to 100 percent), respectively. Event-free survival was also associated with expression of the MRP gene; the five-year rates of event-free survival in the groups with high and low levels of MRP expression were 46 percent (95 percent confidence interval, 25 to 66 percent) and 91 percent (95 percent confidence interval, 82 to 100 percent), respectively. In contrast, there was no difference in either survival (Fig. 2B) or event-free survival with respect to the level of expression of MDR1.

To determine whether the prognostic value of MRP expression was independent of the influence of the N-myc oncogene and other established prognostic indicators, we performed multivariate analyses. When outcome was adjusted for the effect of N-myc amplification, high levels of MRP expression remained a significant indicator of both poor survival (relative hazard, 5.7; 95 percent confidence interval, 1.1 to 30.8) and poor event-free survival (relative hazard, 6.2; 95 percent confidence interval, 1.6 to 24.5) (Table 2). One important result in the multivariate analysis was that N-myc amplification had no prognostic value, a sign that MRP expression accounted for the prognostic value of N-myc amplification. In three separate analyses, the relation between MRP expression and outcome was adjusted for the effects of age, TRK expression, and tumor stage. In each case, high levels of MRP expression remained a significant independent predictor of poor survival and poor event-free survival, with hazard ratios ranging from 4.9 to 9.1. When the four established prognostic indicators — namely, N-myc amplification, age at diagnosis, TRK expression, and tumor stage — were combined with MRP gene expression as variables in the Cox regression model, high levels of MRP expression remained the most powerful indicator of poor survival (relative hazard, 14.9; 95 percent confidence interval, 1.8 to 126.5) and poor event-free survival (relative hazard, 9.7; 95 percent confidence interval, 2.0 to 46.0), independently of all other prognostic indicators (Table 2). With the exception of age, no other variable in this study had significant predictive power in determining outcome, although the upper limits of the confidence intervals for the relative-hazard estimates were too high for us to rule out substantial independent contributions by the other prognostic factors.

Additional analyses supported the strength of the association between MRP expression and outcome. When the MRP values were dichotomized post hoc around the median PCR ratio for the 60 tumors (0.34) rather than the mean ratio, MRP expression remained significantly predictive of both survival (relative hazard, 5.9; 95 percent confidence interval, 1.3 to 27.1) and event-free survival (relative hazard, 5.2; 95 percent confidence interval, 1.5 to 18.4). When the tumors were divided into quartiles according to ascending levels of MRP expression, the cumulative rates of event-free survival for the quartiles were 93, 87, 72, and 38 percent, indicating a correlation between increasing levels of MRP expression and the increasing risk of a poor outcome. This correlation was also found with the Cox proportional-hazards regression model, which showed that the risk of an adverse event increased in proportion to increasing levels of MRP expression; there was a relative hazard of 8.4 (95 percent confidence interval, 1.8 to 38.0) associated with each unit increase in the PCR ratio for this gene.

We also analyzed MRP gene expression in subgroups of patients who were expected on the basis of previously established criteria to have good outcomes. Among patients without N-myc amplification, high levels of MRP

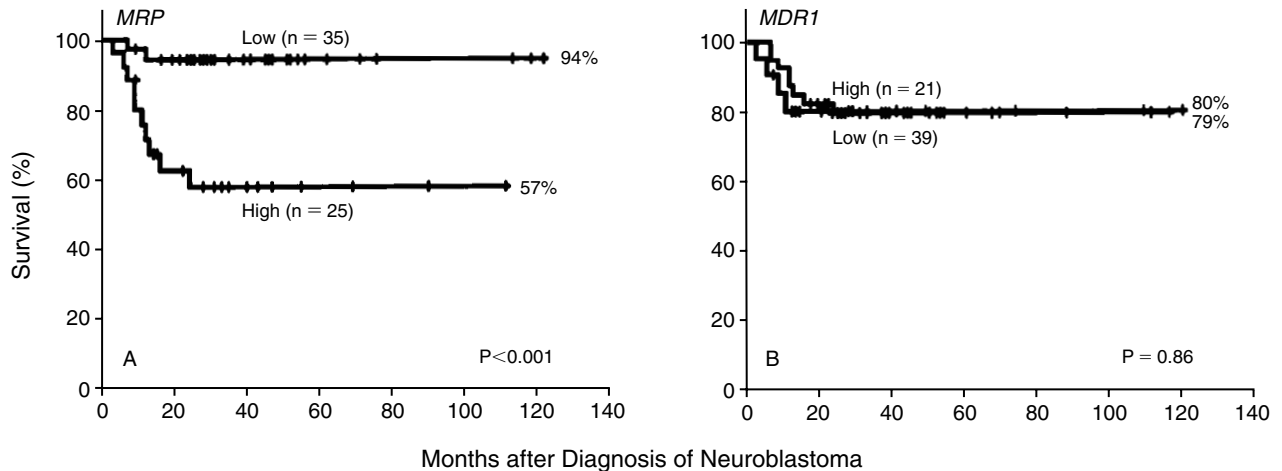


Figure 2. Expression of the *MRP* and *MDR1* Genes and Cumulative Survival in 60 Patients with Neuroblastoma.

The levels of expression of *MRP* (Panel A) and *MDR1* (Panel B) in each primary tumor were determined by a competitive RNA PCR assay, as described in the Methods section. "High" and "low" indicate whether the level of expression of *MRP* or *MDR1* in an individual tumor was higher or lower than the mean PCR ratio calculated for all tumors. P values were determined by the log-rank test. The survival of patients whose tumors expressed high levels of *MRP* was significantly worse than that of patients whose tumors expressed low levels, but *MDR1* expression was not predictive of survival.

expression were associated with significantly reduced rates of event-free survival (relative hazard, 8.9; 95 percent confidence interval, 1.8 to 44.1) (Fig. 3A). Among patients with localized neuroblastoma (Evans stages I, II, and III and Pediatric Oncology Group stages A, B, and C), high levels of *MRP* expression were again associated with significantly reduced event-free survival (relative hazard, 6.1; 95 percent confidence interval, 1.6 to 23.1) (Fig. 3B). These effects were similar in the analysis of overall survival in the two subgroups (relative hazard for patients without *N-myc* amplification, 10.6; 95 percent confidence interval, 1.2 to 95.1; for patients with localized disease, 5.2; 95 percent confidence interval, 1.0 to 26.9).

## DISCUSSION

We found that expression of the multidrug-resistance gene *MRP* at high levels in primary neuroblastoma tumors predicts reduced event-free survival and shorter overall survival in children with this neoplasm. The association between high levels of *MRP* expression and poor outcome was evident both in the overall population of patients and in clinically relevant subgroups. Among the prognostic indicators analyzed, *MRP* gene expression was the most closely associated with outcome. The effect of overexpression of *MRP* appeared to be independent of tumor stage, *TRK* expression, and amplification of the *N-myc* gene. *MRP* expression thus differs from other molecular indicators of outcome in patients with neuroblastoma, such as *TRK* expression and deletions of chromosome 1p,

which have no prognostic value after adjustment in multivariate analysis for the effect of *N-myc* amplification.<sup>37,38</sup> This linkage of prognosis to the *MRP* gene has implications for our understanding of the biology of neuroblastoma and for improving the treatment of patients with this condition.

Amplification of the *N-myc* oncogene is also a predictor of poor outcome in such patients,<sup>3,40</sup> but the molecular basis of the association is unknown. The *N-myc* oncoprotein appears to act as a transcriptional regulator and has been thought perhaps to govern the transcription of critical genes conferring multidrug resistance.<sup>41</sup> Our multivariate analysis, which revealed that *N-myc* amplification had no prognostic value when *MRP* expression was included as a prognostic factor, raises the possibility that the *N-myc* protein regulates expression of the *MRP* gene. This effect could modulate the

Table 2. Multivariate Cox Regression Analysis of Prognostic Factors in Neuroblastoma.\*

FACTOR	SURVIVAL		EVENT-FREE SURVIVAL	
	RELATIVE HAZARD (95% CI)	P VALUE	RELATIVE HAZARD (95% CI)	P VALUE
Adjusted analysis†				
<i>MRP</i> expression	5.7 (1.1–30.8)	0.04	6.2 (1.6–24.5)	0.009
<i>N-myc</i> amplification	2.3 (0.6–8.0)	0.21	1.6 (0.6–4.8)	0.37
Analysis with all factors included in model‡				
<i>MRP</i> expression	14.9 (1.8–126.5)	0.01	9.7 (2.0–46.0)	0.004
Age	8.9 (1.4–56.0)	0.02	2.8 (0.8–10.4)	0.11
<i>TRK</i> expression	2.6 (0.6–10.5)	0.19	2.0 (0.6–7.3)	0.27
<i>N-myc</i> amplification	0.2 (0–1.2)	0.07	0.5 (0.1–2.5)	0.38
Tumor stage§	0	<0.001	0.6 (0.1–2.5)	0.47

\*Prognostic factors are defined as in the notes to Table 1. Relative hazards were calculated as the antilogs of the regression coefficients in the proportional-hazards regression. CI denotes confidence interval.

†Performed by adjusting *MRP* expression for the effect of *N-myc* amplification.

‡Performed after all prognostic factors shown were included in the Cox regression model.

§The relative hazard for tumor stage in the multivariate analysis of survival was 0 because there were no deaths among the patients with favorable tumor stages. The P value in the analysis of survival was obtained by a univariate log-rank test.

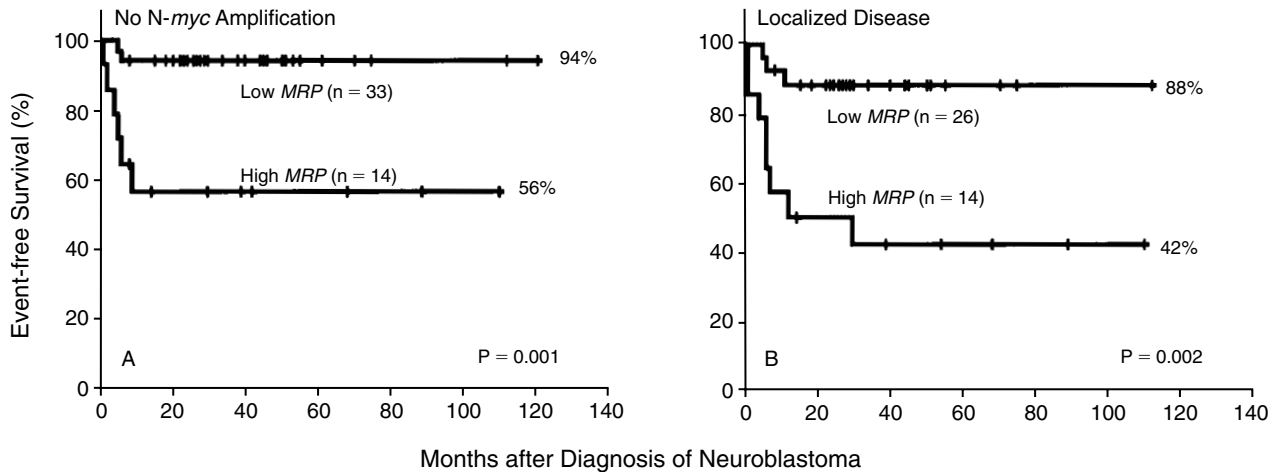


Figure 3. Relation between *MRP* Gene Expression and Outcome in Patients without *N-myc* Amplification and Those with Localized Disease.

The level of *MRP* gene expression in each tumor was determined as described in the legend to Figure 2. Analysis by the log-rank test indicated that in patients whose tumors lacked *N-myc* amplification but expressed high levels of *MRP* (Panel A), event-free survival was significantly worse than in patients whose tumors contained low levels of *MRP*. Similarly, in patients with localized disease (Panel B), tumors expressing high levels of *MRP* were associated with significantly worse event-free survival than were tumors with low levels of *MRP*.

response of neuroblastoma cells to cytotoxic drugs. We previously demonstrated a significant correlation between the expression of the *N-myc* oncogene and that of the *MRP* gene in neuroblastomas and showed that these genes undergo coordinate down-regulation in neuroblastoma cell lines after treatment with retinoic acid.<sup>16</sup> The promoter sequence of the *MRP* gene<sup>42</sup> contains three E-box motifs,<sup>43</sup> which are the consensus DNA-binding sequences of the *myc* family of oncoproteins. However, we do not know whether *N-myc* uses these motifs to influence *MRP* gene expression.

Although both the *MRP* and the *MDR1* genes encode membrane glycoproteins that can function as transporters of multiple drugs, the prognostic value of the two genes differed in this study. Evidence about the contribution of *MDR1* to clinical multidrug resistance in patients with neuroblastoma is contradictory.<sup>5-10</sup> Moreover, several chemotherapeutic agents used to treat neuroblastoma, such as cisplatin and cyclophosphamide, are not substrates for P-glycoprotein.<sup>4</sup> The difference we found in prognostic value between the *MRP* and *MDR1* genes may be explained by the putative physiologic role of *MRP* as an efflux pump for glutathione *S*-conjugates.<sup>44-46</sup> *MRP* has not been shown to mediate resistance to cisplatin or cyclophosphamide, but these drugs do undergo glutathione conjugation.<sup>47,48</sup> It is possible that in their conjugated forms, these drugs could be exported from cells by *MRP*.

A prerequisite for future assessment of the expression of *MRP* is a reproducible standard against which tumor specimens can be compared. The level of *MRP* expression in the SK-N-SH cell line we used fell just below the cutoff value for the uppermost quartile of the PCR ratios for *MRP*. This value (0.56) discriminated

patients with good outcome from those with poor outcome. Future studies may define the relation between intermediate levels of *MRP* expression and clinical outcome, but in evaluating the high *MRP* values that are associated with a poor prognosis, the SK-N-SH cell line may be a useful standard.

A clinical implication of our findings is that compounds capable of inhibiting the action of *MRP*<sup>49-51</sup> may prove therapeutically useful. Our results in a relatively small number of patients with advanced disease need to be confirmed in large prospective studies before any modification of current treatment protocols is considered. It would be feasible to conduct such studies with snap-frozen tumor tissue obtained at diagnosis.

It is plausible that *MRP* influences the outcome in patients with neuroblastoma by directly affecting the response of the tumor to chemotherapy. In a case of aggressive neuroblastoma in which tumor specimens were available both at diagnosis and after treatment, we found that *MRP* expression increased after treatment with cytotoxic drugs (unpublished data). It is also possible that *MRP* influences the aggressiveness of neuroblastoma, its metastatic potential, or both. But regardless of how *MRP* overexpression relates mechanistically to poor outcome, our results suggest that the evaluation of this gene can help in assessing the prognosis of patients with neuroblastoma. Further studies of *MRP* could add to our understanding of the pathogenesis of resistance to chemotherapy in this malignant disease.

We are indebted to the Neuroblastoma Biology Subcommittee of the Pediatric Oncology Group for reviewing and approving this research project and providing samples of neuroblastoma tumors; to Drs. Vivienne Tobias, Ian Kern, and Bruce Currie for assistance in obtaining the tumors from Prince of Wales Children's Hospital; and

to Professor Wayne Hall and Mr. Neil Donnelly, of the National Drug and Alcohol Research Centre, Prince of Wales Hospital, Sydney, Australia, for helpful discussions and assistance with the statistical analysis.

### REFERENCES

- Crist WM, Kun LE. Common solid tumors of childhood. *N Engl J Med* 1991;324:461-71.
- Brodeur GM, Castleberry RP. Neuroblastoma. In: Pizzo PA, Poplack DG, eds. Principles and practice of pediatric oncology. 2nd ed. Philadelphia: J.B. Lippincott, 1993:739-67.
- Brodeur GM, Azar C, Brother M, et al. Neuroblastoma: effect of genetic factors on prognosis and treatment. *Cancer* 1992;70:Suppl:1685-94.
- Roninson IB, ed. Molecular and cellular biology of multidrug resistance in tumor cells. New York: Plenum Press, 1991.
- Chan HSL, Haddad G, Thorner PS, et al. P-glycoprotein expression as a predictor of the outcome of therapy for neuroblastoma. *N Engl J Med* 1991; 325:1608-14.
- Corrias MV, Cornaglia-Ferraris P, Di Martino D, et al. Expression of multiple drug resistance gene, MDR1, and N-myc oncogene in an Italian population of human neuroblastoma patients. *Anticancer Res* 1990;10:897-902.
- Bourhis J, Benard J, Hartmann O, Boccon-Gibod L, Lemerle J, Riou G. Correlation of MDR1 gene expression with chemotherapy in neuroblastoma. *J Natl Cancer Inst* 1989;81:1401-5.
- Goldstein LJ, Fojo AT, Ueda K, et al. Expression of the multidrug resistance, MDR1, gene in neuroblastoma. *J Clin Oncol* 1990;8:128-36.
- Favrot M, Combaret V, Goillot E, et al. Expression of P-glycoprotein restricted to normal cells in neuroblastoma biopsies. *Br J Cancer* 1991;64: 233-8.
- Nakagawara A, Kadomatsu K, Sato S, et al. Inverse correlation between expression of multidrug resistance gene and N-myc oncogene in human neuroblastomas. *Cancer Res* 1990;50:3043-7.
- Cole SPC, Bhardwaj G, Gerlach JH, et al. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* 1992; 258:1650-4.
- Grant CE, Valdimarsson G, Hipfner DR, Almquist KC, Cole SPC, Deeley RG. Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. *Cancer Res* 1994;54:357-61.
- Kruh GD, Chan A, Myers K, Gaughan K, Miki T, Aaronson SA. Expression complementary DNA library transfer establishes *mpr* as a multidrug resistance gene. *Cancer Res* 1994;54:1649-52.
- Slovak ML, Ho JP, Bhardwaj G, Kurz EU, Deeley RG, Cole SPC. Localization of a novel multidrug resistance-associated gene in the HT1080/DR4 and H69AR human tumor cell lines. *Cancer Res* 1993;53:3221-5.
- Zaman GJR, Flens MJ, van Leusden MR, et al. The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. *Proc Natl Acad Sci U S A* 1994;91:8822-6.
- Bordow SB, Haber M, Madafoglio J, Cheung B, Marshall GM, Norris MD. Expression of the multidrug resistance-associated protein (MRP) gene correlates with amplification and overexpression of the N-myc oncogene in childhood neuroblastoma. *Cancer Res* 1994;54:5036-40.
- Brodeur GM, Seeger RC, Barrett A, et al. International criteria for diagnosis, staging, and response to treatment in patients with neuroblastoma. *J Clin Oncol* 1988;6:1874-81.
- Nitschke R, Smith EI, Shochat S, et al. Localized neuroblastoma treated by surgery: a Pediatric Oncology Group study. *J Clin Oncol* 1988;6:1271-9.
- Evans AE, D'Angio GJ, Randolph J. A proposed staging for children with neuroblastoma: children's cancer study group A. *Cancer* 1971;27:374-8.
- Seeger RC, Brodeur GM, Sather H, et al. Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. *N Engl J Med* 1985;313:1111-6.
- Telford DJ, Kavallaris M, White L, Norris MD, Brian MJ, Stewart BW. Association of N-myc amplification with neuroblastoma: the Australian and New Zealand experience. *J Paediatr Child Health* 1992;28:58-63.
- Shimada H, Chatten J, Newton WA Jr, et al. Histopathologic prognostic factors in neuroblastic tumors: definition of subtypes of ganglioneuroblastoma and an age-linked classification of neuroblastomas. *J Natl Cancer Inst* 1984; 73:405-16.
- Bernard JL, Philip T, Zucker JM, et al. Sequential cisplatin/VM-26 and vincristine/cyclophosphamide/doxorubicin in metastatic neuroblastoma: an effective alternating non-cross-resistant regimen? *J Clin Oncol* 1987;5:1952-9.
- McCowage GB, Vowels MR, Shaw PJ, Lockwood L, Mameghan H. Autologous bone marrow transplantation for advanced neuroblastoma using teniposide, doxorubicin, melphalan, cisplatin and total body irradiation. *J Clin Oncol* 1995;13:2789-95.
- Joshi VV, Cantor AB, Brodeur GM, et al. Correlation between morphologic and other prognostic markers of neuroblastoma: a study of histologic grade, DNA index, N-myc gene copy number, and lactic dehydrogenase in patients in the Pediatric Oncology Group. *Cancer* 1993;71:3173-81.
- Castleberry RP, Kun LE, Shuster JJ, et al. Radiotherapy improves the outlook for patients older than 1 year with Pediatric Oncology Group stage C neuroblastoma. *J Clin Oncol* 1991;9:789-95.
- Nitschke R, Smith EI, Altshuler G, et al. Postoperative treatment of nonmetastatic visible residual neuroblastoma: a Pediatric Oncology Group study. *J Clin Oncol* 1991;9:1181-8.
- Bowman LC, Castleberry RP, Altshuler G, et al. Therapy based on DNA index (DI) for infants with unresectable and disseminated neuroblastoma (NB): preliminary results of the Pediatric Oncology Group "Better Risk" Study. *Med Pediatr Oncol* 1990;18:364. abstract.
- Castleberry RP, Cantor AB, Green AA, et al. Phase II investigational window using carboplatin, iproplatin, ifosfamide, and epirubicin in children with untreated disseminated neuroblastoma: a Pediatric Oncology Group study. *J Clin Oncol* 1994;12:1616-20.
- Strother D, Shuster JJ, McWilliams N, et al. Results of Pediatric Oncology Group protocol 8104 for infants with stages D and DS neuroblastoma. *J Pediatr Hematol Oncol* 1995;17:254-9.
- Noonan KE, Beck C, Holzmayer TA, et al. Quantitative analysis of MDR1 (multidrug resistance) gene expression in human tumors by polymerase chain reaction. *Proc Natl Acad Sci U S A* 1990;87:7160-4.
- Holzmayer TA, Hilsenbeck S, Von Hoff DD, Roninson IB. Clinical correlates of MDR1 (P-glycoprotein) gene expression in ovarian and small-cell lung carcinomas. *J Natl Cancer Inst* 1992;84:1486-91.
- Haber M, Madafoglio J, Bordow SB, et al. Expression of retinoic acid-responsive genes in primary neuroblastomas. In: Evans AE, Biedler JL, Brodeur GM, D'Angio GJ, Nakagawara A, eds. Advances in neuroblastoma research 4: proceedings of the Sixth Symposium on Advances in Neuroblastoma Research held in Philadelphia, Pennsylvania, May 13-15, 1993. Vol. 385 of Progress in clinical and biological research. New York: Wiley-Liss, 1994:245-51.
- Marshall GM, Cheung B, Stacey KP, et al. Increased retinoic acid receptor gamma expression suppresses the malignant phenotype and alters the differentiation potential of human neuroblastoma cells. *Oncogene* 1995;11:485-91.
- Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-9.
- Gilbert J, Norris MD, Haber M, Kavallaris M, Marshall GM, Stewart BW. Determination of N-myc gene amplification in neuroblastoma by differential polymerase chain reaction. *Mol Cell Probes* 1993;7:227-34.
- Nakagawara A, Arima-Nakagawara M, Scavarda NJ, Azar CG, Cantor AB, Brodeur GM. Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma. *N Engl J Med* 1993; 328:847-54.
- Christiansen H, Sahin K, Berthold F, Hero B, Terpe H-J, Lampert F. Comparison of DNA aneuploidy, chromosome 1 abnormalities, MYCN amplification and CD44 expression as prognostic factors in neuroblastoma. *Eur J Cancer* 1995;31A:541-4.
- Look AT, Hayes FA, Shuster JJ, et al. Clinical relevance of tumor cell ploidy and N-myc gene amplification in childhood neuroblastoma: a Pediatric Oncology Group study. *J Clin Oncol* 1991;9:581-91.
- Schwab M. Molecular cytogenetics of human neuroblastoma. *Biochim Biophys Acta* 1992;1114:43-50.
- Bénard J, Bourhis J, de Vathaire F, et al. Prognostic value of MDR1 gene expression in neuroblastoma: results of a multivariate analysis. In: Evans AE, Biedler JL, Brodeur GM, D'Angio GJ, Nakagawara A, eds. Advances in neuroblastoma research 4: proceedings of the Sixth Symposium on Advances in Neuroblastoma Research, held in Philadelphia, Pennsylvania, May 13-15, 1993. Vol. 385 of Progress in clinical and biological research. New York: Wiley-Liss, 1994:111-6.
- Zhu Q, Center MS. Cloning and sequence analysis of the promoter region of the MRP gene of HL60 cells isolated for resistance to adriamycin. *Cancer Res* 1994;54:4488-92.
- Blackwell TK, Huang J, Ma A, et al. Binding of myc proteins to canonical and noncanonical DNA sequences. *Mol Cell Biol* 1993;13:5216-24.
- Jedlitschky G, Leier I, Buchholz U, Center M, Keppler D. ATP-dependent transport of glutathione S-conjugates by the multidrug resistance-associated protein. *Cancer Res* 1994;54:4833-6.
- Muller M, Meijer C, Zaman GJR, et al. Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione S-conjugate transport. *Proc Natl Acad Sci U S A* 1994;91:13033-7.
- Leier I, Jedlitschky G, Buchholz U, Cole SPC, Deeley RG, Keppler D. The MRP gene encodes an ATP-dependent export pump for leukotriene C<sub>4</sub> and structurally related conjugates. *J Biol Chem* 1994;269:27807-10.
- Tew KD. Glutathione-associated enzymes in anticancer drug resistance. *Cancer Res* 1994;54:4313-20.

48. Ishikawa T, Ali-Osman F. Glutathione-associated *cis*-diamminedichloroplatinum(II) metabolism and ATP-dependent efflux from leukemia cells: molecular characterization of glutathione-platinum complex and its biological significance. *J Biol Chem* 1993;268:20116-25.
49. Gekeler V, Boer R, Ise W, Sanders KH, Schachtele C, Beck J. The specific bisindolylmaleimide PKC-inhibitor GF 109203X efficiently modulates MRP-associated multiple drug resistance. *Biochem Biophys Res Commun* 1995; 206:119-26.
50. Gekeler V, Ise W, Sanders KH, Ulrich W-R, Beck J. The leukotriene LTD<sub>4</sub> receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. *Biochem Biophys Res Commun* 1995;208:345-52.
51. Versantvoort CHM, Broxterman HJ, Lankelma J, Feller N, Pinedo HM. Competitive inhibition by genistein and ATP dependence of daunorubicin transport in intact *MRP* overexpressing human small cell lung cancer cells. *Biochem Pharmacol* 1994;48:1129-36.
- 

#### IMAGES IN CLINICAL MEDICINE

Images in Clinical Medicine, a weekly *Journal* feature, presents clinically important visual images, emphasizing those a doctor might encounter in an average day at the office, the emergency department, or the hospital. If you have an original unpublished, high-quality color or black-and-white photograph representing such a typical image that you would like considered for publication, send it with a descriptive legend to Kim Eagle, M.D., University of Michigan Medical Center, Division of Cardiology, 3910 Taubman Center, Box 0366, 1500 East Medical Center Drive, Ann Arbor, MI 48109. For details about the size and labeling of the photographs, the requirements for the legend, and authorship, please contact Dr. Eagle at 313-936-5275 (phone) or 313-936-5256 (fax), or the *New England Journal of Medicine* at [images@edit.nejm.org](mailto:images@edit.nejm.org) (e-mail).