

Multidrug Resistance-Associated Protein 1 (MRP1) Expression in Neuroblastoma Cell Lines and Primary Tumors

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Background and Procedure. MRP1 expression by neuroblastomas was evaluated by Northern blot analysis in 21 cell lines and 90 primary untreated tumors. Cytotoxicity assay in cell lines was performed for five anticancer drugs used in treating neuroblastoma. **Results.** MRP1 expression did not correlate with drug resistance or with MYCN RNA expression in cell lines. MRP1 expression was higher in drug-sensitive cell lines established after chemotherapy relative to cell lines at diagnosis, but highly drug-resistant cell lines showed low MRP1 expression. Positive expression of MRP1

RNA in primary tumors was associated with a poorer survival relative to MRP1-negative tumors. However, MRP1 expression levels did not correlate with age, stage, MYCN amplification, or MYCN expression, and higher MRP1 expression was not associated with a worse outcome. **Conclusions.** In neuroblastoma, positive MRP1 RNA expression at diagnosis has prognostic significance, but high drug resistance is conferred by mechanisms other than MRP1. *Med. Pediatr. Oncol.* 35:619–622, 2000. © 2000 Wiley-Liss, Inc.

Key words: neuroblastoma, multidrug resistance, prognostic factors, MYCN oncogene, MRP1

INTRODUCTION

Multidrug resistance (MDR) is a major obstacle in chemotherapy for patients with cancer. The membranous proteins of the ATP-binding cassette transporter family (ABC transporters) are thought to confer MDR, and a number of investigators have studied the relation between expression of ABC transporters and treatment outcome [1]. In neuroblastoma, it has been reported that high expression of multidrug resistance-associated protein 1 (MRP1) RNA was associated with MYCN amplification and poor treatment outcome [2], although another group did not find a significant correlation between MRP1 expression and prognosis [3]. This study was performed to determine the following in a panel of cell lines and primary untreated tumors.

1. Whether expression of MRP1 RNA correlated with resistance to drugs used in neuroblastoma chemotherapy.
2. Whether expression of MRP1 in tumors at diagnosis would have any prognostic significance.

MATERIALS AND METHODS

Primary tumor tissue from 90 untreated neuroblastoma patients was obtained from the Children's Cancer Group (CCG) Neuroblastoma Resource Laboratory; 37 tumors were in low stage (I, II, and IV-S), and 53 were high stage (III and IV). Amplified MYCN gene was detected in 18 tumors (20.0%); 50.6% were diagnosed after

the age of 12 months. All patients were treated according to risk group on Children's Cancer Studies, primarily CCG-3891 and CCG-3881, and tumors were collected for research under protocols approved by institutional review boards.

A panel of 21 cell lines was analyzed in this study. Cell culture and cytotoxicity assay were performed as previously described [4]. Cell lines were classified ac-

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ording to the phase of disease when established. Seven were established at diagnosis before chemotherapy (DX), 8 were at progressive disease on chemotherapy (PD), and 6 were at relapse after myeloablative chemotherapy with auto-BMT (PD-BMT). Cytotoxicity assays for doxorubicin, etoposide, cisplatin, carboplatin, and melphalan were performed using the DIMSCAN assay system, and the lethal drug concentrations for 90% of the cells (LC_{90} values) were calculated [4]. A cell line was considered to be resistant to the drug when the LC_{90} value for that drug was higher than a clinically achievable level. In this study, drug-resistant cell lines were defined as those cell lines resistant to more than half of drugs tested.

The detection and quantification of RNA expression for MRP1 and MYCN was performed by Northern blot analysis as previously described [5], and standardized to β -actin expression with the highest expressing cell line (SMS-KCNR) defined as 100. MYCN expression was quantified only in cell lines.

Correlation of MRP1 expression with LC_{90} values and MYCN expression was determined by the Spearman correlation coefficient test. Comparison of the mean or median MRP1 expression levels among groups was performed by two-sided Student *t*-test or Wilcoxon nonparametric two-sample test. Associations between MRP1 expression and patients' characteristics were analyzed by the χ^2 test or Fisher exact test. The Kaplan-Meier method was used to evaluate event-free and overall survival curves, and the log-rank statistic was used to compare survival between subgroups of patients [6]. Multivariate analysis was performed using the regression method of Cox [7].

RESULTS

MRP1 Expression in Cell Lines

MRP1 RNA expression was observed in all cell lines. There was no significant correlation between the level of MRP1 expression and LC_{90} values, and the median MRP1 values were not statistically different between cell lines sensitive and resistant to each drug. The number of drug-resistant cell lines were five of eight at PD, and four of six at PD-BMT. All DX cell lines were drug-sensitive. The median expression level of MRP1 RNA tended to be higher in cell lines established after chemotherapy relative to those established at diagnosis, but this was not statistically different for all 21 cell lines (32.6 at PD and PD-BMT, 18.0 at DX, $P = 0.101$). However, for drug-sensitive cell lines, the cell lines derived after chemotherapy expressed significantly higher MRP1 than did cell lines established before chemotherapy (64.4 versus 18.0, $P = 0.015$). In five pairs of cell lines from the same patients before and after chemotherapy, those cell lines established after exposure to chemotherapy expressed 3.4, 2.0, 1.0, 1.5, and 1.5 times higher MRP1 RNA than the corresponding cell lines at diagnosis.

MYCN RNA expression was found in 18 of 21 cell lines. Consistent with previous observations [8], in five pairs from the same patients, the expression level of MYCN RNA increased after chemotherapy. Comparing the expression level of MRP1 and MYCN RNA in 21 cell lines revealed no statistical correlation ($r = 0.420$, $P = 0.058$).

MRP1 Expression in Primary Tumors

Positive expression of MRP1 RNA by Northern blotting (expression level >0.1) was seen in 53 of 90 tumors (58.9%). Positive expression of MRP1 was significantly associated with high stage ($P < 0.001$), age older than 1 year at diagnosis ($P = 0.043$), MYCN gene amplification ($P = 0.016$), and MYCN expression ($P = 0.012$). However, a comparison of the mean value of MRP1 as a cutoff failed to show a significant correlation with any those factors.

Follow-up data were available in 85 tumors as shown in Figure 1A. Positive expression of MRP1 was statistically associated with a lower event-free ($P = 0.012$) and overall survival ($P = 0.016$). In stage III and IV disease, where chemotherapy was a part of therapy, the expression of MRP1 was still associated with a lower overall survival ($P = 0.041$). However, in stages I, II, and IV-S tumors, overall survival was not different between MRP1-positive and MRP1-negative tumors. To estimate the dose effect of MRP1 expression on survival, we used the mean value (10.0) as a cutoff. Overall and event-free survival of 20 tumors with higher MRP1 expression was not statistically different from the 65 tumors with lower MRP1 ($P = 0.36$ and 0.37 , respectively). When tumors were divided into three groups according to the expression level of MRP1, the overall survival for patients with tumors showing high, intermediate, or no expression was 59.9%, 49.7%, and 81.1% at 5 years, respectively (Fig. 1B). Thus, positive expression of MRP1 correlated with a lower probability of survival, but there was no evidence that higher levels of MRP1 at diagnosis conferred a worse prognosis.

DISCUSSION

In spite of the well-documented function of MRP1 as an efflux pump for drugs [1], there are few studies to show that a reduced intracellular drug concentration is the main mechanism of treatment failure in cancer chemotherapy. In a panel of neuroblastoma cell lines, the expression level of MRP1 did not correlate with the LC_{90} values of any drugs tested. However, drug-sensitive cell lines established after chemotherapy (PD or PD-BMT) expressed higher MRP1 RNA than did those at diagnosis, although drug-resistant cell lines showed low expression levels of MRP1. The same trend was seen in five pairs of cell lines established from identical patients before and

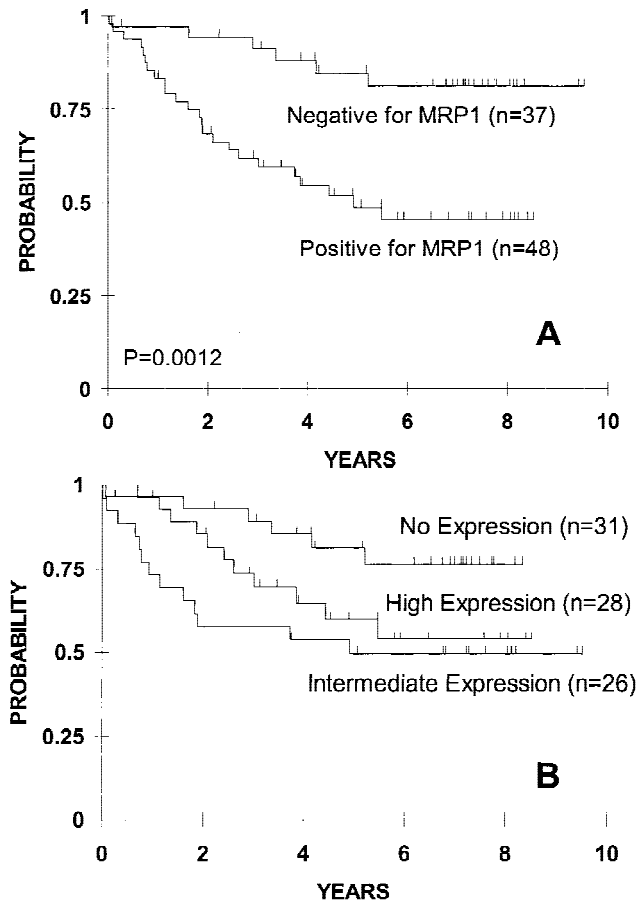


Fig. 1. **A:** Comparison of overall survival (OS) by MRP1 expression. Overall 5-year survival of patients with MRP1-positive tumors (MRP1 level >0.1) was 48.6% compared to 84.6% for patients with tumors negative for MRP1 RNA expression. **B:** Comparison of OS by MRP1 expression level. Patients were divided into three groups according to MRP1 expression level: no expression, intermediate expression (expression level: 0–4.6), and high expression (expression level >4.6) group. Overall 5-year survival of each group was 81.1%, 49.7%, or 59.9%, respectively.

after chemotherapy. The cell lines established after exposure to chemotherapy tended to express higher MRP1 than did the corresponding cell lines established before treatment. However, this trend was not seen for those cell lines that were highly drug resistant after chemotherapy. These results suggest that a selection for those cells with high expression of MRP1 may occur in drug-sensitive tumors, implying that MRP1 confers a survival advantage, but that high drug resistance in neuroblastoma is conferred by mechanisms other than MRP1. One such mechanism is a loss of p53 function, which correlated with high drug resistance in the panel of cell lines studied here [9].

Several groups have reported a significant correlation between MRP1 and MYCN expression [2,3,10–12]. On the other hand, the transfection of the *MYCN* gene into neuroblastoma cells has been reported to not enhance

MRP1 expression [3]. In five pairs of cell lines from the same patients, MYCN expression was enhanced after chemotherapy, and we also observed higher MRP1 expression in drug-sensitive postchemotherapy cell lines. However, looking at all 21 cell lines, there was no statistical correlation between MYCN and MRP1 RNA expression. Positive MRP1 expression was observed more frequently in primary tumors with *MYCN* gene amplification or with detectable MYCN RNA expression, but the mean MRP1 expression level was not statistically different, regardless of *MYCN* gene status. Our preliminary data with a MYCN nonexpressing neuroblastoma cell line transfected with *MYCN* failed to show that overexpressing MYCN increased MRP1 expression (H. Goto et al., unpublished data). Taken together, these findings suggest that MYCN expression does not regulate MRP1 expression, but may correlate with MRP1 expression by an indirect mechanism.

In primary untreated tumors, the detectable expression of MRP1 significantly correlated with other prognostic factors and with a lower probability of survival. Thus, the positive expression of MRP1 is likely to be one characteristic of high-risk neuroblastoma. In a Cox multivariate analysis, the positive expression of MRP1 still showed an independent prognostic significance in overall survival ($P = 0.046$). However, the effect of MRP1 on survival did not depend on expression level. The fact that a higher expression of MRP1 does not always relate with poor survival is consistent with the results in cell lines, where some lines with enhanced MRP1 expression are still drug sensitive, whereas other cell lines with low MRP1 expression were highly drug resistant.

Low-stage neuroblastomas (I, II, and IV-S) are thought to have a biology that is distinct from that of high-stage tumors. In this study, a higher MRP1 expression than the mean value was detected in 5 of 37 low-stage tumors (13.5%), which was not significantly different from the 28.3% stage III and IV tumors with MRP1 levels greater than the mean. In those low-stage tumors, the expression of MRP1 did not have any prognostic significance. However, positive expression of MRP1 correlated with a lower overall survival in stage III and IV tumors ($P = 0.041$). Thus, MRP1 expression appears to confer a survival advantage for tumor cells during chemotherapy, and MRP1 may only affect clinical outcome in tumors treated with chemotherapy. This implies that MRP1 requires other biologic features responsible for aggressive tumor behavior before its expression has a significant effect on survival. Finally, our data suggest that the survival advantage conferred by MRP1 in neuroblastoma is likely to be replaced during tumor progression with other mechanisms that confer high-level drug resistance.

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