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Original Paper

The Prognostic Value of *MDR*1 Gene Expression in Primary Untreated Neuroblastoma

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The contribution of *MDR*1 gene expression to the biology of childhood neuroblastoma is unclear. To clarify the role of *MDR*1 in this malignancy, we examined the relationship between *MDR*1 expression and patient outcome in subsets of 60 primary untreated neuroblastomas for which *MYCN* gene copy number and expression of the multidrug resistance-associated-protein (MRP) gene had been previously characterised. In contrast to *MRP* gene expression, *MDR*1 expression was lower in tumours with *MYCN* gene amplification compared with those without amplification. Strong correlations between *MDR*1 and *MRP* gene expression, and between *MDR*1 and *MYCN* gene expression, were observed in tumours lacking *MYCN* gene amplification ($P < 0.0005$). In these single-copy tumours, very high *MDR*1 gene expression was significantly associated with poor outcome ($P < 0.05$). Very high *MDR*1 expression was also strongly predictive of poor outcome in older children ($P < 0.0001$), but not in infants. These findings suggest a clinical role for the *MDR*1 gene in specific subgroups of primary neuroblastoma. © **1997 Elsevier Science Ltd.**

Key words: multidrug resistance, *MDR*1, multidrug resistance-associated protein (MRP), neuroblastoma, *MYCN* oncogene, polymerase chain reaction (PCR)
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INTRODUCTION

THE DEVELOPMENT of resistance to multiple cytotoxic drugs is the major cause of treatment failure in childhood neuroblastoma. Such multidrug resistance is particularly common in patients whose tumours display amplification of the *MYCN* oncogene [1]. Although *MYCN* gene amplification is one of the most powerful predictors of poor outcome identified for neuroblastoma [2, 3], it is not known how *MYCN* influences response to chemotherapeutic agents. The two best characterised mechanisms of multidrug resistance identified to date involve the *MDR*1 gene, encoding P-glycoprotein (Pgp) [4] and the more recently described multidrug resistance-associated protein (MRP) gene [5]. Both MRP and Pgp are ATP-dependent membrane transport proteins which, *in vitro*, are capable of conferring resistance to a number of natural product drugs, including the anthracyclines, epipodophyllotoxins and *vinca* alkaloids [4, 6, 7].

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We have recently shown that expression of the *MRP* gene is a powerful predictor of outcome in neuroblastoma [8]. In our study, *MDR*1 did not predict for outcome. However, the contribution of the *MDR*1 gene to the drug-resistant phenotype of neuroblastoma remains controversial, with evidence both for [9–11] and against [12–14] a direct role. In an attempt to identify the basis for this controversy, we studied the relationship between *MDR*1 expression and patient outcome in subsets of a cohort of 60 primary untreated neuroblastomas for which *MYCN* gene copy number and *MRP* gene expression had been previously characterised. The results indicate that the characteristics of the neuroblastoma population under study may influence the prognostic significance of this gene.

PATIENTS AND METHODS

Patients and tumour specimens

The 60 primary untreated neuroblastomas employed in this study have been described previously [8] and were obtained either from the Neuroblastoma Tumor Bank of the

Pediatric Oncology Group, U.S.A., or from the Sydney Children's Hospital (formerly the Prince of Wales Children's Hospital), Sydney, Australia. All clinical disease stages were represented, and tumours had previously been subjected to Southern blot analysis to determine the number of copies of the *MYC*N oncogene per haploid genome [2, 15]. Tumours were classified as having *MYC*N amplification where more than three *MYC*N copies were present. Outcome measures studied were survival, defined as time from diagnosis to death, and event-free survival, defined as time from diagnosis to the first major event (relapse, failure to achieve remission or death).

Analysis of gene expression by the polymerase chain reaction (RNA-PCR)

Isolation of total cytoplasmic RNA, synthesis of complementary DNA (cDNA) and the competitive polymerase chain reaction (RNA-PCR) assay have been described previously [8, 16]. Aliquots of cDNA corresponding to 50 ng of RNA were amplified for 30 cycles and each target gene sequence (*MRP*, *MDR*1 or *MYC*N) was co-amplified with a control gene sequence (β_2-*microglobulin*) using gene-specific oligonucleotide primers, described elsewhere [16]. Following triplicate PCR analyses, and polyacrylamide gel electrophoresis of PCR products, the level of expression of each target gene in each tumour was determined by densitometric scanning of photographic negatives, and was expressed relative to the level of control β_2-*microglobulin* gene expression.

Statistical analysis

The relationship between levels of expression of various target genes was analysed by linear regression. Differences between groups of tumour specimens in terms of their PCR ratios for a given target gene were assessed by Student's *t*-test, using two-sided *P* values. For the survival analyses, the *MDR*1 PCR ratio of each individual tumour was categorised as 'low' or 'high' according to one of several procedures. Values of *MDR*1 gene expression were dichotomised either around the mean PCR ratio obtained from all 60 tumour specimens [8], or where specifically indicated, around the median, 80th percentile or 90th percentile PCR ratio. Survival analyses were performed according to the method of Kaplan and Meier, and comparisons of outcome between subgroups were performed by the log-rank test for univariate comparisons, using two-tailed tests. Results are expressed as mean ± standard error, and survival probabilities and relative hazards are given with 95% confidence intervals.

RESULTS

MRP, MDR*1 and* MYC*N gene expression in primary neuroblastoma*

The relationship between *MRP* and *MDR*1 gene expression in the 60 primary neuroblastoma specimens was analysed by linear regression (Figure 1). This analysis, which related the PCR ratio for *MRP* expression to that of the *MDR*1 gene for each sample, revealed a highly significant correlation between these two parameters ($R = 0.43$; $P = 0.0005$). Thus, although *MRP* gene expression had been highly predictive of clinical outcome in this cohort of patients, while *MDR*1 expression had not [8], these two parameters nevertheless appeared to have a close statistical relationship.

To understand the basis of this paradoxical result, the relationship between expression of the *MRP* and *MDR*1 genes was further examined in subsets of tumours. When tumours were grouped according to the presence or absence of amplification of the *MYC*N oncogene, *MRP* gene expression was significantly higher in the 13 tumours having *MYC*N gene amplification compared to the 47 tumours without amplification (Figure 2a). In contrast, the level of *MDR*1 expression was lower in the tumours with *MYC*N amplification, although this difference failed to achieve statistical significance (Figure 2b; $P = 0.089$). To determine whether a different relationship between *MRP* and *MDR*1 gene expression existed in these two subsets of tumours, separate linear regression analyses were performed. In tumours without *MYC*N gene amplification, the relationship between MRP and *MDR*1 gene expression (Figure 3a) was even more powerful than that which had been observed in the overall study population ($R = 0.732$; $P < 0.0001$). However, no such relationship was observed in the tumours having *MYC*N gene amplification (Figure 3b; $R = 0.03$; $P = 0.94$).

We have previously reported that *MRP* gene expression correlates with expression of the *MYC*N oncogene, both *in vivo* and *in vitro* [16]. Based on these findings and on the results shown in Figure 2, it would therefore be anticipated that *MDR*1 gene expression would be highly correlated with the level of expression of the *MYC*N oncogene, but only in tumours without *MYC*N gene amplification. To test this hypothesis, separate linear regression analyses were performed, relating expression of the *MDR*1 and *MYC*N genes, in the subsets of tumours either with or without *MYC*N gene amplification (Figure 4). A highly significant correlation was indeed observed between *MDR*1 and *MYC*N gene expression ($R = 0.52$; $P = 0.0002$) in tumours lacking *MYC*N gene amplification. In contrast, *MYC*N and *MDR*1 gene expression were unrelated in the *MYC*N amplified tumours ($R = 0.04$; $P = 0.89$).

MDR*1 gene expression and outcome*

Having established a strong relationship between *MDR*1 gene expression and expression of both the *MYC*N and *MRP* genes in tumours lacking *MYC*N gene amplification, it was important to determine the prognostic influence of *MDR*1

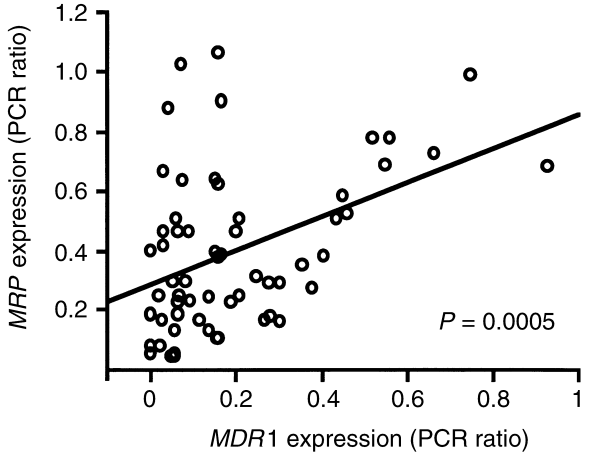

Figure 1. Linear regression analysis indicating a significant correlation between expression of the *MDR*1 and *MRP* genes in the overall study population of 60 primary untreated neuroblastoma tumours.

gene expression in such tumours. The effect on patient outcome of *MDR*1 gene expression in tumours without *MYC*N gene amplification was initially determined according to previous methodology [8], by dichotomising *MDR*1 values around the mean PCR ratio (0.199) for the 60 tumours in the overall study population. No difference in either survival or event-free survival was observed (Table 1) between patients whose tumours had either high or low levels of *MDR*1 gene expression. Similar results were obtained when *MDR*1 values were dichotomised, *post hoc*, around the median (50th percentile) PCR ratio for the 60 tumours (0.152).

Subsequent examination of the distribution of *MDR*1 expression levels in the overall study population revealed a skewed distribution, such that a subset of tumours displayed particularly high expression of the *MDR*1 gene. In order to determine whether very high *MDR*1 expression was associated with poor outcome, Kaplan–Meier survival analyses were performed in which the *MDR*1 PCR ratio of an individual tumour was classified as 'high' only if it exceeded the 80th, or alternatively the 90th, percentile PCR ratio for *MDR*1 expression in the 60 tumours. When these analyses were performed on the overall study population of 60 tumours, there was no significant difference in either survival

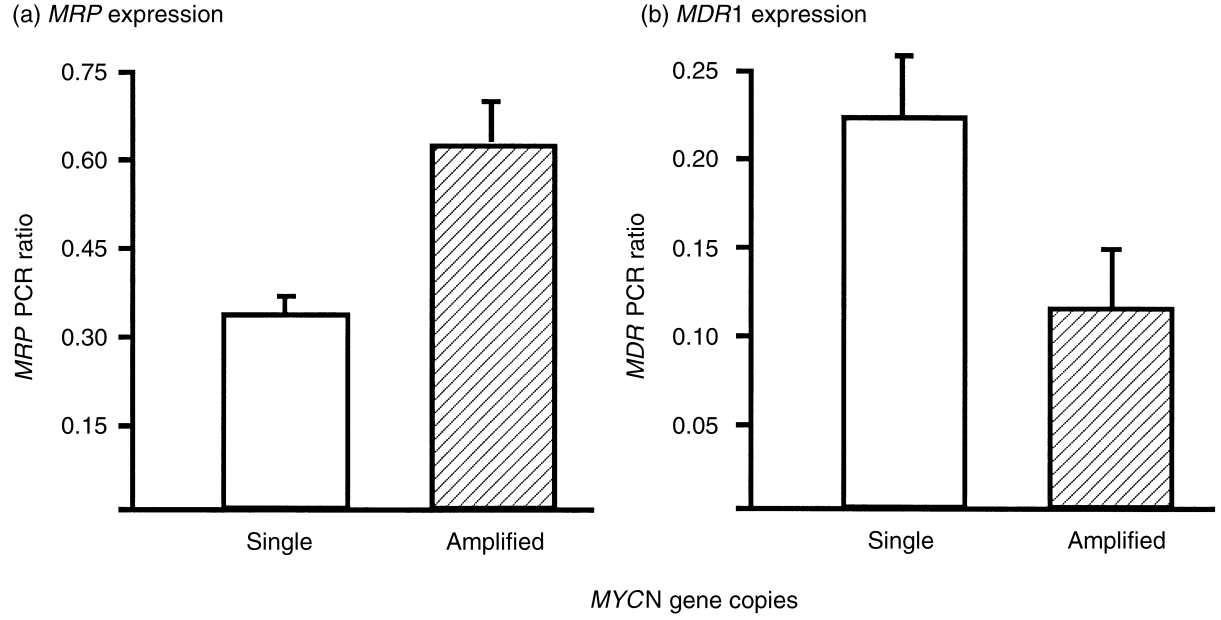

Figure 2. Expression of the (a) *MRP* and (b) *MDR*1 genes in primary neuroblastoma tumours displaying either single or multiple copies of the *MYC*N oncogene. Columns, mean; bars, SE.

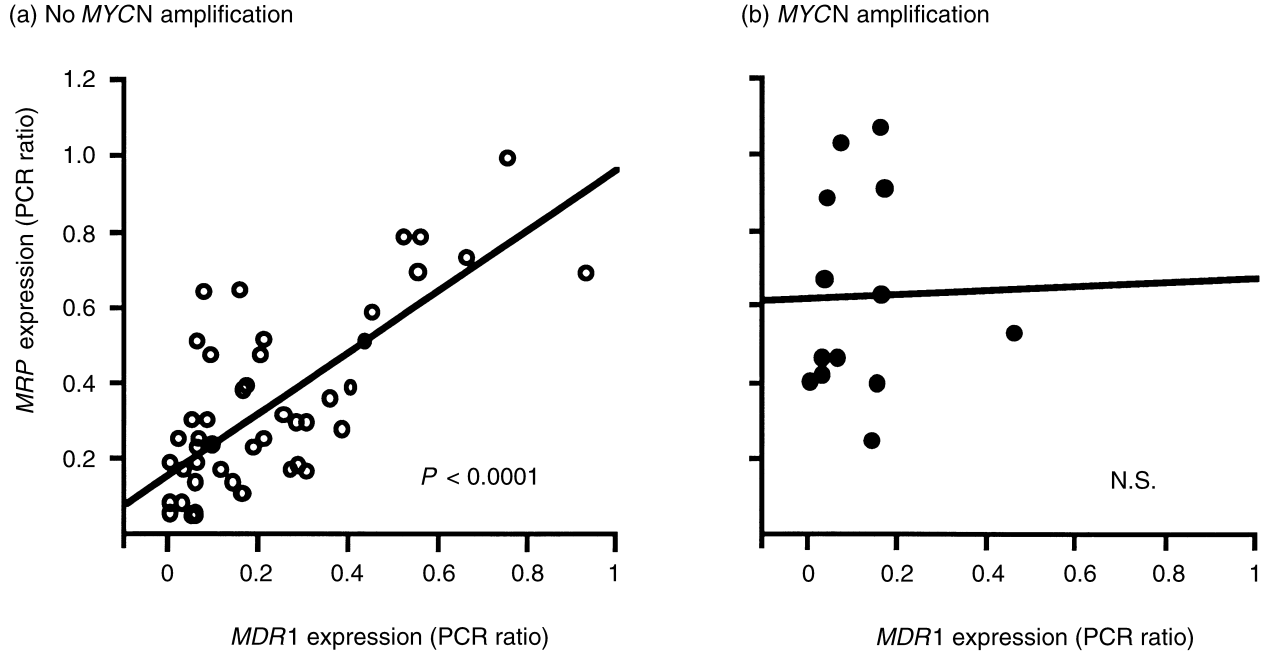

Figure 3. Correlation between expression of the *MDR*1 and *MRP* genes in neuroblastoma tumours having either (a) single ($n = 47$) or (b) multiple ($n = 13$) copies of the *MYC*N oncogene. Linear regression analyses indicated a highly significant correlation between expression of these two genes only in tumours lacking *MYC*N gene amplification.

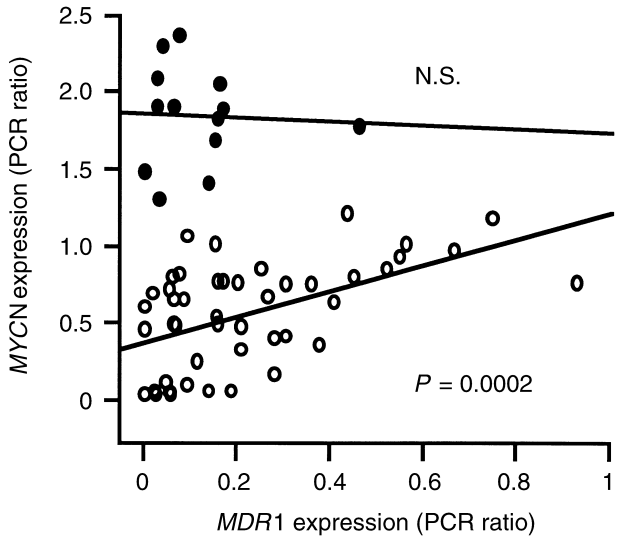

Figure 4. Correlation between expression of the *MDR*1 and *MYCN* genes in neuroblastoma tumours displaying either single (○) or multiple (●) copies of the *MYCN* oncogene. Linear regression analyses indicated a highly significant relationship between *MDR*1 and *MYCN* gene expression only in tumours lacking *MYCN* gene amplification.

or event-free survival of patients with respect to the level of expression of *MDR*1. Thus, irrespective of the cut-off point used to discriminate between 'low' and 'high' *MDR*1 gene expression, the level of expression of this gene did not affect outcome in the overall study population (Table 1).

However, when the prognostic effect of particularly high levels of *MDR*1 gene expression was studied in the subset of tumours without *MYCN* gene amplification, a significant association between high expression and poor outcome was observed (Figure 5, Table 1). This association was apparent using either the 80th or 90th percentile PCR ratio (0.307 and 0.489, respectively) as the cut-off point to discriminate low from high gene expression (Table 1), and using either survival (Figure 5a) or event-free survival (Figure 5b) as the outcome measure. For event-free survival, the relative hazard associated with very high *MDR*1 gene expression was 4.35 (95% confidence interval, 1.08–17.54) and 5.71 (95% confidence interval, 1.35–24.39), using the 80th and 90th percentile PCR ratio as the cut-off point, respectively.

To determine whether very high *MDR*1 expression would also be prognostic in patients who, on the basis of previously established criteria, were likely to have poor outcomes, we performed Kaplan–Meier survival analyses relating *MDR*1 expression to outcome in the subset of patients aged greater

Table 1. *Relationship between MDR*1 *gene expression and outcome in neuroblastoma populations: effect of altering the cut-off point discriminating between 'high' and 'low' MDR*1 *gene expression*

Study population	Cut-off point (*MDR*1 PCR ratio)			
	50th percentile (0.152)	Mean (0.199)	80th percentile (0.307)	90th percentile (0.489)
	P values*			
All patients ($n = 60$)	N.S.	N.S.	N.S.	N.S.
Single-copy *MYCN* ($n = 47$)	N.S.	N.S.	0.029	0.028
Age >1 year ($n = 31$)	0.034	0.040	0.0003	<0.0001

**P* values were derived from log-rank tests which compared cumulative survival of patients whose tumours had 'high' or 'low' *MDR*1 expression, respectively, defined as indicated in the table. In all cases, when these analyses were repeated using event-free survival as the outcome measure, identical results were obtained. N.S., not significant ($P > 0.05$).

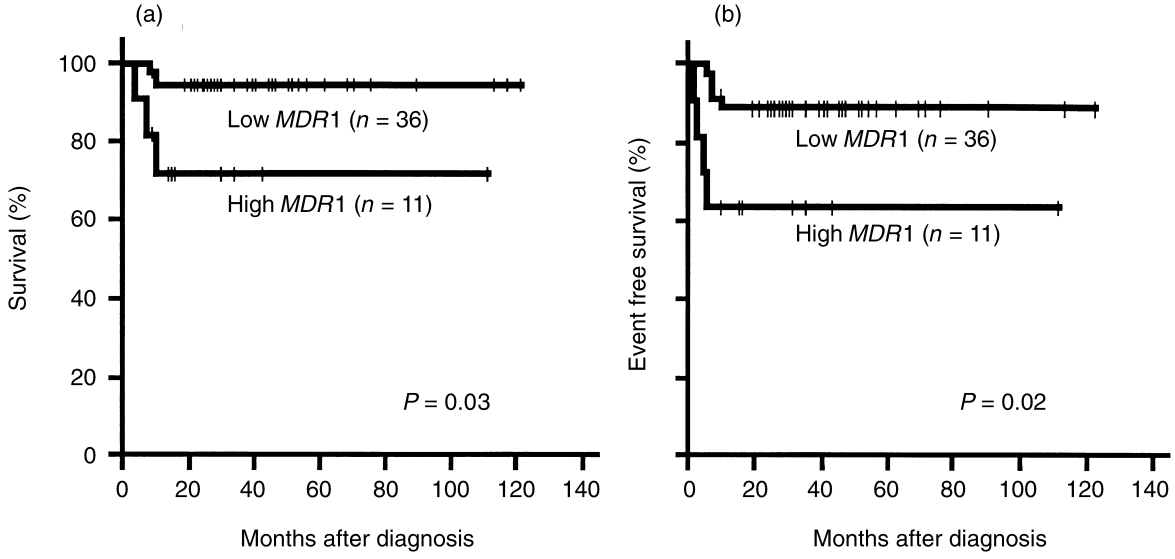

Figure 5. Expression of the *MDR*1 gene and cumulative survival (a) or event-free survival (b) in patients without *MYCN* gene amplification. The cut-off point used to discriminate between tumours having 'high' or 'low' *MDR*1 gene expression was the 80th percentile PCR ratio. Tick marks indicate the length of follow-up of individual patients.

than 1 year at diagnosis. Again, using either the 80th or 90th PCR percentile ratio as the cut-off point, a strong association was observed between high levels of *MDR*1 expression and reduced survival (Table 1). With the 80th percentile as the cut-off point, the 5-year cumulative survival rates of the groups of patients aged greater than one year at diagnosis having high and low *MDR*1 gene expression, respectively, were 20% (95% confidence interval, 0–55%) and 77% (95% confidence interval, 60–93%). The 5-year rates of event-free survival in these respective groups of patients were 20% (95% confidence interval, 0–55%) and 71% (95% confidence interval, 53–90%). For event-free survival, the relative hazard associated with high *MDR*1 gene expression was 7.63 (95% confidence interval, 2.13–27.03) and 12.05 (95% confidence interval, 1.98–71.43), using the 80th and 90th percentile PCR ratio as the cut-off point, respectively. In contrast to the analyses performed either on the overall study population or on patients without *MYC*N gene amplification, significant associations were observed between high *MDR*1 and poor outcome of patients aged greater than 1 year at diagnosis even when *MDR*1 values were designated as high or low on the basis of dichotomising around either the mean or median *MDR*1 PCR ratios of the 60 tumours (Table 1). Thus, irrespective of the cut-off point used to categorise tumours into those having high and low *MDR*1 expression, significantly worse outcome was associated with higher levels of *MDR*1 expression in this subset of patients. No significant associations between *MDR*1 expression and outcome were observed for infants aged less than 1 year at diagnosis.

DISCUSSION

Increased expression of the *MDR*1 gene has been associated with increased risk of treatment failure in several human malignancies including lymphoblastic and myeloid leukaemias [17–19], soft tissue sarcomas [20] and osteosarcoma [21]. However, the role of the *MDR*1 gene in mediating multidrug resistance in neuroblastoma is unclear. Chan and associates [9] demonstrated that Pgp expression in neuroblastoma independently predicted for poor outcome, but other studies of *MDR*1 expression in this malignancy have failed to confirm these findings [8, 12, 14]. Controversy regarding the contribution of *MDR*1 to the chemoresistant phenotype of this disease was heightened by the study of Favrot and associates [13], which reported that Pgp expression in neuroblastoma was restricted to the normal infiltrating cells of the stroma. While a number of studies have shown a marked increase in *MDR*1 expression following chemotherapy [10, 11], such increased *MDR*1 expression following drug treatment might well be due to chemotherapy-induced differentiation of the tumour cells [14]. Thus, several laboratories, including our own [16], have demonstrated that in neuroblastoma cell lines induced to differentiate by exposure to retinoic acid, *MDR*1 expression increases in parallel with other markers of neuronal differentiation [22]. However, whether such increased expression of the *MDR*1 gene correlates with increased drug resistance is not at all clear, since Bates and associates [22] reported that increased expression of *MDR*1 in the differentiated neuroblastoma cells was not associated with the expected decrease in accumulation of a number of cytotoxic drugs. As a result of these disparate data, the contribution of the *MDR*1 gene to either drug resistance or to patient outcome in this disease remains ill-defined.

In the present study, *MDR*1 gene expression failed to predict for outcome in the overall study population, regardless of the cut-off point used to divide the tumours into high and low categories. Nevertheless, a close relationship was apparent between *MDR*1 gene expression and expression of the MRP gene which we have recently shown to be a powerful indicator of outcome for neuroblastoma [8]. This apparent paradox was resolved by demonstrating distinct patterns of expression of the *MDR*1 and MRP genes in tumours with amplification of the *MYC*N oncogene compared to those without. Thus, high MRP expression but low *MDR*1 expression was observed in tumours with *MYC*N amplification and expression of these two genes was not correlated in this subset of tumours. In view of the powerful prognostic significance of both *MYC*N gene amplification [2] and high MRP gene expression [8] in neuroblastoma, failure of high *MDR*1 expression to correlate with either of these parameters in the *MYC*N amplified tumours accounts for the lack of predictive power of *MDR*1 gene expression in the overall study population. In the tumours without *MYC*N amplification, however, expression of *MDR*1 correlated with both MRP and *MYC*N gene expression and in this subset of tumours, the highest levels of *MDR*1 expression did predict for outcome. Thus, the characteristics of the individual study population can influence the prognostic power of *MDR*1 gene expression. This finding was highlighted by the particularly powerful prognostic significance of *MDR*1 expression in older children, which was not evident in infants.

The finding of a strong correlation between expression of the MRP, *MDR*1 and *MYC*N genes in tumours lacking *MYC*N gene amplification is consistent with the hypothesis that the *MYC*N oncogene, a transcriptional regulator, might contribute to the chemoresistant phenotype of neuroblastoma by regulating expression of critical drug-resistance genes, including *MDR*1 [23] and MRP [8, 24]. In tumours with *MYC*N amplification, *MYC*N and MRP were highly correlated [24], but the correlation between expression of *MDR*1 and either MRP or *MYC*N was lost. Discrepant results regarding the relationship between *MYC*N and *MDR*1 gene expression in neuroblastoma have previously been described. Thus, while it is well established that retinoic acid-induced downregulation of *MYC*N gene expression is accompanied by increased *MDR*1 gene expression [16, 22] and activation of the *MDR*1 promoter [25], it has also been reported that during metastatic dissemination in mice, *MYC*N and *MDR*1 genes are coactivated [26]. While neither the factors regulating *MDR*1 expression in neuroblastoma, nor the role of *MYC*N in this process, are understood, it is apparent that these regulatory processes are disrupted in tumours having amplification of the *MYC*N oncogene.

In addition to the composition of individual study populations, the present findings suggest that the prognostic significance of the *MDR*1 gene may also be influenced by the definition of high versus low expression. Previous investigations into the role of *MDR*1 in neuroblastoma biology have varied widely, not only in the assay methods used for *MDR*1 determination, but also in the definition of high *MDR*1 expression, as well as in the tumour material used for study (i.e. diagnosis versus post-treatment specimens) and these factors have undoubtedly contributed to the conflicting data in this field. The present findings, suggesting prognostic significance for *MDR*1 gene expression in certain subpopulations of neuroblastoma patients, will need to be confirmed

prospectively and moreover, highlight the need to ensure representative sample selection when conducting *MDR*1 expression studies in this aggressive disease.

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