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Expression of *mdr*-1/P-glycoprotein in Human Neuroblastoma

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Increased expression of the* mdr-*1 gene encoding the drug efflux pump P-glycoprotein is a well-established mediator of acquired drug resistance* in vitro, *and a similar role has been hypothesized* in vivo *in human malignancy. Because expression of* mdr-*1 is increased in neuroblastoma cell lines by differentiating agents, the authors hypothesized a similar correlation with differentiation* in vivo *in neuroblastomas. In 12 tumors from 11 patients, total RNA analysis demonstrated no correlation with differentiation, but a correlation could be detected in the cell-based methods of analysis. The very primitive 'stroma'-poor, poorly differentiated neuroblastomas had low levels of* mdr-*1/P-glycoprotein. The intermediate grades had higher levels of expression and although heterogeneity of differentiation appeared within these tumors, both primitive and more differentiated cells expressed the gene at comparable levels within the tumor. One very well-differentiated neuroblastoma, a ganglioneuroma, had no detectable expression in the neurofibrillary material, but demonstrated expression in adjacent large ganglionic cells. Thus* mdr-*1/P-glycoprotein expression increased with increasing differentiation among tumors, and was present in ganglionic cells in the most well-differentiated tumor. The three tumors with the highest levels of expression were obtained from patients who received preoperative chemotherapy. (Am J Pathol 1991, 139:305–315)

The study of multidrug resistance *in vitro* has demonstrated that exposure of cells to a single agent can confer resistance to several structurally unrelated natural products, including the vinca alkaloids, the epipodophyllotoxins, the anthracyclines, and actinomycin D.[1–5] These 'multidrug-resistant' cell lines display overexpression of the *mdr*-1/P-glycoprotein gene, which encodes a 170,000 dalton glycoprotein originally described by Ling.[4,6,7] On the cell membrane, P-glycoprotein serves as an energy-dependent drug efflux pump, which decreases drug accumulation in cell lines in which this protein is overexpressed.[8–10] Various compounds, including calcium channel blockers, can compete with chemotherapeutic agents for binding to P-glycoprotein, thereby increasing drug accumulation in the cell and cytotoxicity.[11,12]

Increased expression of *mdr*-1/P-glycoprotein has been established to mediate acquired drug resistance *in vitro*, and there is limited evidence to support a similar role *in vivo* in human malignancy. Further P-glycoprotein expression may play an important role in primary drug resistance in the tumors in which it is found before the administration of chemotherapy. These tumors include many in which the natural products are ineffective, such as kidney, colon, and adrenocortical cancer.[13] Expression has been documented also in neuroblastoma, a more chemoresponsive tumor.[13–15] Most patients with neuroblastoma typically respond to treatment regimens that include the natural products, but subsequently relapse and are refractory to further therapy.[16,17] Whether this treatment failure is due to P-glycoprotein–mediated resistance or to other mechanisms has not been determined.

Drug resistance in neuroblastoma has been extensively studied *in vitro*. Cell lines established from neuroblastoma demonstrate a range of drug sensitivity.[18] Selection of neuroblastoma cell lines in the presence of actinomycin D or vincristine results in overexpression of P-glycoprotein.[19] Interestingly with the development of multidrug resistance in these cells, one observes a more differentiated phenotype with cell flattening and lengthening of neuritic processes, along with decreased tumorigenicity.[20] The results suggest, however, that differentiation is incomplete, as evidenced by an increase in anchorage-independent growth in two cell lines, and an increase in N-*myc* expression in one. Thus the onset of *in vitro* multidrug resistance in neuroblastoma results in a phenotype suggesting a limited pattern of differentiation.

A correlation between expression of *mdr*-1/P-

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glycoprotein and differentiation has been observed in unselected neuroblastoma cell lines *in vitro*.[14] Expression was increased with treatment of the cell lines with the differentiating agent, retinoic acid. However the onset of *mdr*-1 expression after differentiation was not associated with detectable alterations in drug accumulation. In the present study, we examined the expression of *mdr*-1 in tumors obtained from 11 patients with neuroblastoma to determine whether expression would be associated with a differentiated phenotype. We analyzed 12 neuroblastoma tissues by RNA *in situ* hybridization, immunohistochemistry, and RNase protection assay. In each case, some level of *mdr*-1 gene expression could be observed, but high levels such as those observed in *in vitro* drug resistant cell lines were less common. A correlation of expression with differentiation also could be demonstrated.

Materials and Methods

Cell Lines

Controls for *mdr*-1/P-glycoprotein expression included the drug-sensitive parental epidermoid carcinoma cell line, KB 3-1, and a drug-resistant subline, KB 8-5.[21] These lines are well described, and the sixfold increase in vinblastine resistance in KB 8-5 appears to be entirely due to expression of P-glycoprotein.[21] The SK-N-SH neuroblastoma cell line was obtained from June Biedler.[22] Cell lines were carried in Roswell Park Memorial Institute medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum, and 100 IU of penicillin and 50 μg of streptomycin per milliliter. Treatment of the SK-N-SH cell line *in vitro* with 5×10^{-6} mol/l retinoic acid was performed in tissue culture dishes containing slides coated with 2% triethoxysilane (Sigma Chemical Co., St. Louis, MO) in preparation for RNA *in situ* hybridization.[23,24]

Neuroblastoma Tissues

Tissues were obtained from the Cooperative Human Tissues Network, Columbus Childrens Hospital, Columbus, Ohio. Tissues were obtained by surgical excision, and were frozen and shipped in dry ice. They were stored at −70°C until use. For RNA *in situ* hybridization, tissues were broken while still frozen, embedded in optimal cutting temperature medium, and 8-μ sections placed on prepared slides. For Northern analysis and RNase protection, tissues were homogenized in guanidinium isothiocyanate, and centrifuged over a cesium chloride gradient.[25]

Hybridization Probes

Two *mdr*-1/P-glycoprotein probes were used: 1) For RNA *in situ* hybridization, a 1.4-Kb cDNA containing sequences from the middle third of P-glycoprotein in a pGEM vector was used for riboprobe synthesis.[26,27] 2) For RNase protection, a 1-Kb *Pst* I genomic fragment from the 5′ end of the gene[28] subcloned into pGEM was used for synthesis of riboprobe. The predominate fragment protected is 130 base pairs (bp). Fragments of slightly varying length are presumed to be protected by mRNA arising from alternate mRNA start sites.

RNA Extraction

Total cellular RNA was extracted from cells by homogenizing in guanidine isothiocyanate followed by centrifugation over a cesium chloride cushion. Total RNA was electrophoresed in 1% agarose, 6% formaldehyde gels, which were subsequently stained with 2 μg/ml ethidium bromide to allow inspection of the quantity and quality of RNA.[25]

RNase Protection Assay

Protection of 1×10^6 cpm labeled riboprobe from ribonuclease digestion by hybridization with 20 μg total RNA was performed.[29] Samples were denatured at 85°C for 5 minutes, followed by overnight incubation at 45°C in 80% Formamide (Fluka, Buchs, Switzerland), 0.4 mol/l NaCl, 40 mmol/l (millimolar) Pipes, 1 mmol/l ethylenediaminetetra-acetic acid, 2 mmol/l $LiCl_2$. Subsequently, the samples were digested for 1 hour at 30°C with 40 μg/ml RNase A and 28 U/ml RNase T_1 (Boehringer Mannheim, Indianapolis, IN); and then for 15 minutes with 15 μg/ml proteinase K. After extraction, samples were separated on a 6% polyacrylamide gel at 1500 volts for 2 to 3 hours.

RNA In Situ *Hybridization*

Microscope slides were coated in 2% triethoxysilane (Sigma) before plating of cells in tissue culture media. Slides bearing cells or tissue sections were fixed 2 minutes in 4% paraformaldehyde. *In situ* hybridization was carried out as previously described after pretreatment with 0.25% acetic anhydride and immersion in 0.1 mol/l Tris/0.1 mol/l glycine.[30] Slides then were prehybridized for 5 minutes at 52°C in 2× SSC/50% formamide/10 mmol/l dithiothreitol (DTT). Riboprobes for *in situ* hybridization were labeled to a high specific activity using ^{35}S-uridine 5′ (alphathio)-triphosphate (Amersham, Arlington

Heights, IL). For 5 hours at 50°C, 1.5 × 10^6 cpm probe was hybridized to the cells in 50% formamide (Fluka), 10% dextran sulfate (Sigma) 2× SSC, 2 mg/ml bovine serum albumin (Boehringer-Mannheim), 1000 μg/ml *Escherichia coli* tRNA (Sigma), 1000 μg/ml Herring sperm DNA (Sigma), and 50 mmol/l DTT (Sigma). Washes were performed at 50°C in 50% formamide/2× SSC/10 mmol/l DTT followed by treatment with 100 μg/ml RNase A for 30 minutes at 37°C. After dehydration in an ascending ethanol series, slides were dipped in Kodak NTB emulsion (Eastman-Kodak, Rochester, NY) for a 2-week autoradiographic exposure. After development, total tissue autoradiograms were obtained using Hyperfilm-βmax (Amersham) at 5°C for 4 weeks. The specificity of the signal for each tumor was demonstrated by negative results with a sense probe, a riboprobe sequence transcribed from the 1.4-kb *mdr*-1 cDNA subcloned into the pGEM vector in the reverse orientation. Each tumor was hybridized in duplicate in four separate experiments.

P-glycoprotein Immunohistochemistry

Frozen sections were lyophilized overnight, then fixed for 10 minutes in 3.7% formaldehyde and washed in phosphate-buffered saline. Subsequently slides were treated as previously described[31] and then incubated for 1 hour at 23°C in either 10 μg/ml MRK-16 antibody, which is specific for P-glycoprotein,[32] or in 10 μg/ml IgG Γ2a (Coulter Immunology, Hialeah, FL). A peroxidase-conjugated anti-mouse antibody (Jackson Immunoresearch Laboratories, Westgrove, PA) was used to detect MRK-16 binding. Each tumor was examined in duplicate in a minimum of three separate experiments.

Histologic Grading

For the purpose of this study, the histologic grading ranged from I to IV, and was based on certain histologic features derived from Shimada et al's classification scheme.[33] Specifically 'stroma'-poor (grades I and II), and 'stroma'-rich categories were recognized, and the degree of neuroblastic differentiation was expressed as 'well' or 'poorly differentiated' histology (Table 1). The presence of a certain percentage (more than 5% in Shimada and colleagues' scheme) of differentiating neuroblastic or ganglionic cells was not required in the present study to place the tumor in the differentiated histology because exact evaluation of neuroblastic differentiation is more difficult on frozen than paraffin sections. Easily identified differentiating neuroblasts were required, however, for inclusion in the category of differentiated histology. The same criteria of cytologic differentiation were used both in 'stroma'-poor and 'stroma'-rich tumors. As a rule, however, the cytologic differentiation in the 'stroma'-rich tumors was higher altogether, when compared with that of the 'stroma'-poor tumors. In general, grade I tumors in this scheme correspond to the conventional neuroblastoma, grade II to diffuse (imperfect) ganglioneuroblastoma, grade III to immature (composite) ganglioneuroblastoma, and grade IV to ganglioneuroma. The nodular 'stroma'-rich category of Shimada and colleagues' scheme could not be included in our grading system, because it requires gross evaluation of the specimen for discrete, grossly visible nodules, which was impossible in the present material. Moreover the mitotic/karyorrhetic index, also used in Shimada and colleagues' scheme, was not easily evaluable on the frozen tissues. Finally, because the goal of this study was to evaluate expression of P-glycoprotein in relation to degree of histologic differentiation, age was not considered as a factor in our grading system, in contrast to Shimada and colleagues' scheme, which serves different purposes.

Table 1. *Histologic Grading of Neuroblastoma*

Grade I
Cellular aggregates of tumor cells with fibrovascular septae, but not axonal/Schwannian bundles, define the "stroma" poor category. None or very few differentiating neuroblasts are present.
Grade II
The tumor exhibits the same stroma-poor pattern as above, but also a fair number of differentiating neuroblasts or true ganglionic cells.
Grade III
There is a fair amount of 'stroma,' ie, axonal processes ensheathed by Schwann cells, and ganglionic cells. However aggregates of primitive neuroblastic cells are present as well. The overall degree of histologic differentiation is usually higher than that of grade II neuroblastoma, but certainly less than that seen in the next grade, which corresponds to ganglioneuroma.
Grade IV
Stroma-rich tumor with terminally differentiated ganglion and Schwann cells.

Results

Neuroblastoma tissues were evaluated by RNase protection analysis, RNA *in situ* hybridization, and immunohistochemistry. Representative hematoxylin and eosin (H&E) sections from all four histologic grades are shown in Figure 1. Despite the use of frozen sections, evaluation of cytologic differentiation was not difficult, and the 'stroma'-poor and -rich tumors were easily recognizable. The four histologic grades are described in Materials and Methods and in Table 1. The 12 tumors were obtained from 11 patients; biopsies were taken on one patient before and after chemotherapy. Five of the patients had received prior chemotherapy. Table 2 summarizes the

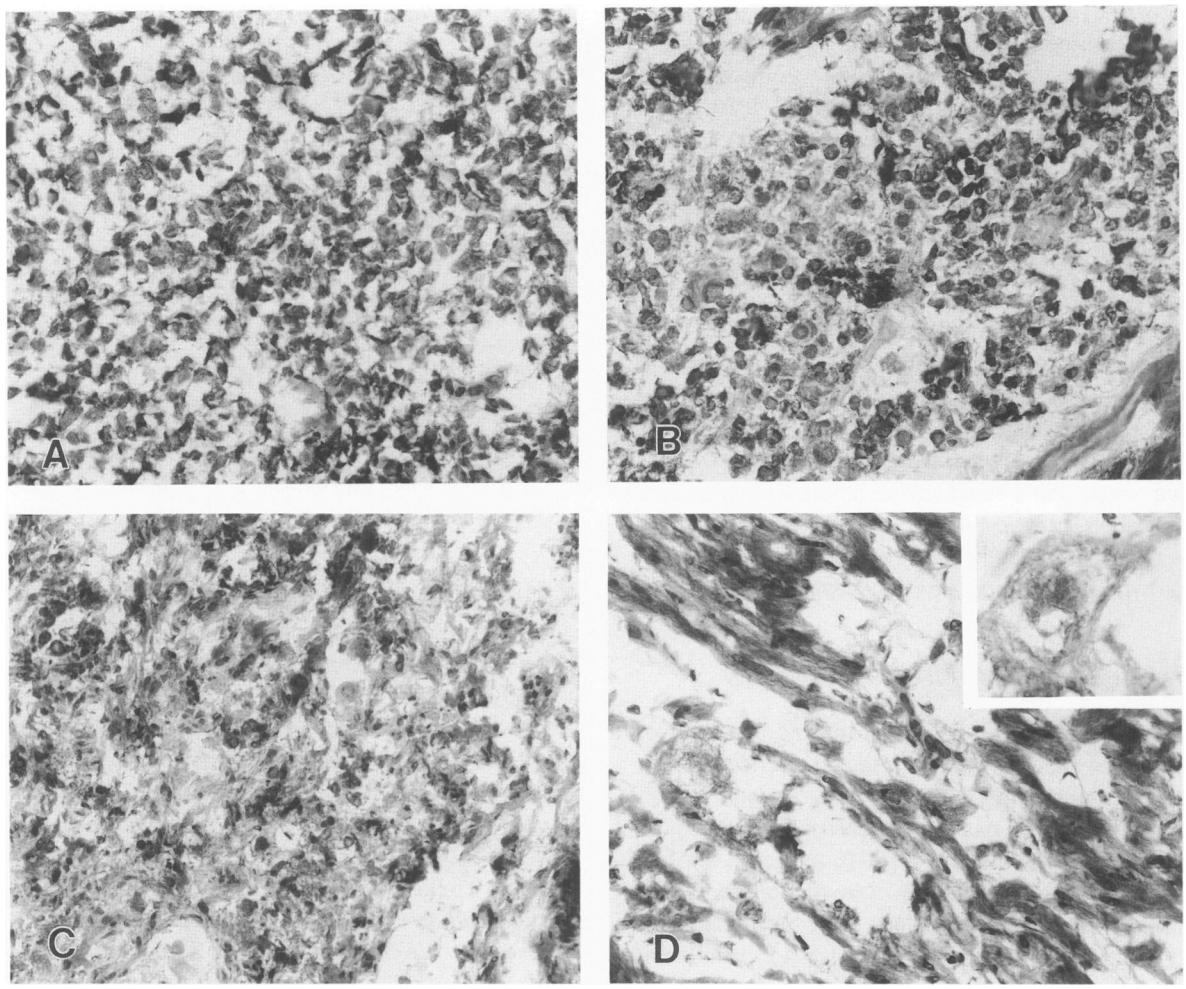


Figure 1. *H&E sections representative of the four histologic grades used in the studies. Except where noted, the magnification is ×250.* **A** *is the grade I, 11-D02 tumor.* **B** *is the 'stroma'-poor, differentiating grade II, 08-Z01 tumor. The tumor cells are larger and exhibit more ample cytoplasm.* **C** *is the 'stroma'-rich grade III, 09-EE02 tumor.* **D** *is the well-differentiated, 'stroma'-rich grade IV, 04-N01 tumor, which corresponds to a conventional ganglioneuroma. The inset shows a ganglionic cell, magnification ×500.*

age, stage, primary site, treatment, and survival for each patient. The degree of tumor differentiation and the *mdr*-1/P-glycoprotein expression data, which will be shown in succeeding figures, is included as well.

Figure 2 demonstrates the expression of *mdr*-1 by RNase protection in the nine tumors in which sufficient tissue was obtained for RNA harvest. The template used is a genomic fragment from the 5′ end of the gene; *mdr*-1 mRNA transcripts protect a 130-bp fragment, which can be seen in all of the tumors, with some at higher levels than others. In this analysis, no obvious correlation with differentiation could be demonstrated, because 09-EE02 (c), a 'stroma'-rich, poorly differentiated grade III tumor, demonstrates lower levels than 11-I04 (d), which is a 'stroma'-poor, differentiating grade II tumor.

Because the heterogeneous composition of neuroblastomas could obscure a correlation between *mdr*-1/P-glycoprotein expression and differentiation, we sought to examine *mdr*-1 expression at a cellular level using RNA *in situ* hybridization and immunohistochemistry. The sensitivity of the RNA *in situ* analysis was established in cell culture models of neuroblastoma, in which we had previously demonstrated by Northern analysis increased *mdr*-1 expression after addition of differentiating agents. In the *in situ* analysis shown in Figure 3, increased mRNA expression in SH-SY5Y cells after treatment with retinoic acid for 3 to 5 days is seen as an increase in the average number of grains per cell (B), compared with control (A). The levels after retinoic acid treatment are similar to those seen in the KB 8-5 drug-resistant cell line that was obtained by chronic exposure to colchicine (C) and is fourfold to sixfold resistant to adriamycin and vinblastine compared with its parental subline, KB 3-1.

Having established the sensitivity of the RNA *in situ* analysis in cell culture, this approach was applied to the patient samples. The value of this method of analysis is evident in Figure 4, which shows the results observed in

Table 2. *Correlation Between* mdr-*1/P-glycoprotein Expression and Differentiation*

Name	Age	Stage	Primary Site*	Prior Rx*	Differentiation	*mdr*-1†	P-glycoprotein†	Survival (mo)
08-U01	2 m	III/IV	S	N	II	1+	2+	42
03-U02	4 m	III	R	N	III	1+	1+	36
04-I08	9 w	II	S	N	II	1+	0	32
04-N01	3 y	IV	S	Y	IV	0/4+§	0/4+§	8‖
07-I01‡	8 y	III	S	N	I	1+	1+	28
07-I22	3 y	IV	R	N	I	1+	0-1+	31
08-Z01	7 y	IV	S	Y	II	4+	4+	16‖
09-EE02	15 m	IV	R	Y	III	3+	3+	27
11-I04‡	8 y	III	S	Y	II	1+	2+	24
11-I07	3 w	III	R	N	I	2+	1+	22
11-D02	7 y	IV	S	Y	I	1+	1-2+	28
12-D01	22 m	III	S	N	II	1+/4+§	1+	2‖

* Primary site is noted as either suprarenal (S) or retroperitoneal (R); and prior Rx indicates yes (Y) or no (N) prior therapy.

† Expression of *mdr*-1 and P-glycoprotein was assigned a level from lowest, 1+ to highest, 4+. Expression of *mdr*-1 or P-glycoprotein was not correlated with age, stage, site or survival; but both were associated with prior therapy. Previous treatment was positvely correlated with expression of *mdr*-1, Pearson's correlation coefficient 0.617 ($P = 0.032$) and likewise P-glycoprotein, Pearson's correlation coefficient 0.812 ($P = 0.001$).

‡ Denotes the single patient from whom tissue was obtained before and after chemotherapy, with 11-I04 designating the post-treatment tissue.

§ Indicates two levels of expression in the tumor. 12-D01 is shown in Figure 4 and 04-N01 in Figure 5.

‖ Denotes death of patient. Patient 12-D01 died 2 months after diagnosis, in partial response, with acute respiratory distress syndrome.

a 'stroma'-poor, well-differentiated tumor. The upper panel is a bright field photograph, whereas the lower panel is a photograph of the dark field results. Very low levels of expression are found in both primitive and more differentiated areas in the majority of the tumor, with a focal area of intense *mdr*-1 expression. In addition to the bright- and dark-field photomicrographs of the focal region of high expression, an autoradiogram of the entire tumor section obtained after RNA *in situ* hybridization is shown between the photographs. The area appeared as a nodule that disappeared on serial sections, and was not represented in the sections used for immunohistochemistry, which showed only the low-level P-glycoprotein staining present in the majority of the tissue. Multiple sections through the nodule, however, showed intense *mdr*-1 expression. No histologic differences could be determined between the area of intense expression and the rest of the tumor. Although this patient had not received any chemotherapy before surgery, such a focal area could survive and emerge as the predominant cell type after chemotherapy.

The striking heterogeneity observed in Figure 4 was uncommon. More commonly, morphologic heterogeneity was demonstrated with consistent patterns of P-glycoprotein expression. It was consistently observed that when expression was detected, it was present in both the primitive and well-differentiated ganglionic cells of a given area. The majority of tissues showed some degree of immunohistochemical staining for P-glycoprotein and some mRNA signal by RNA *in situ* hybridization.

Figure 5 demonstrates the results obtained by both

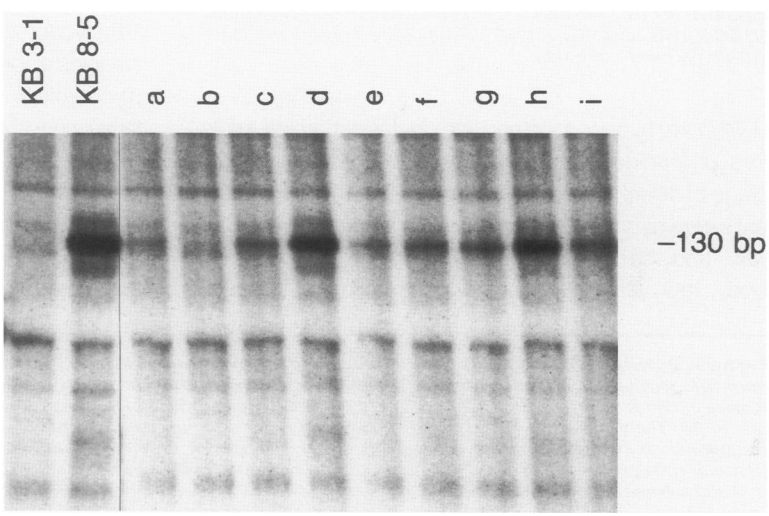


Figure 2. *RNase protection analysis of mdr-1 mRNA in the neuroblastoma tissues. The expected 130-bp fragment can be seen in all of the neuroblastoma tissues, but absent in the drug-sensitive KB 3-1 cell line. The letters are associated with tumors* **a**: *07-I01,* **b**: *12-D01,* **c**: *09-EE02,* **d**: *11-I04, 3: 03-U02,* **f**: *04-I08,* **g**: *11-I07,* **h**: *08-Z01,* **i**: *09-Z01 (this tumor studied by RNase protection only). No correlation with grade could be made with this analysis.*

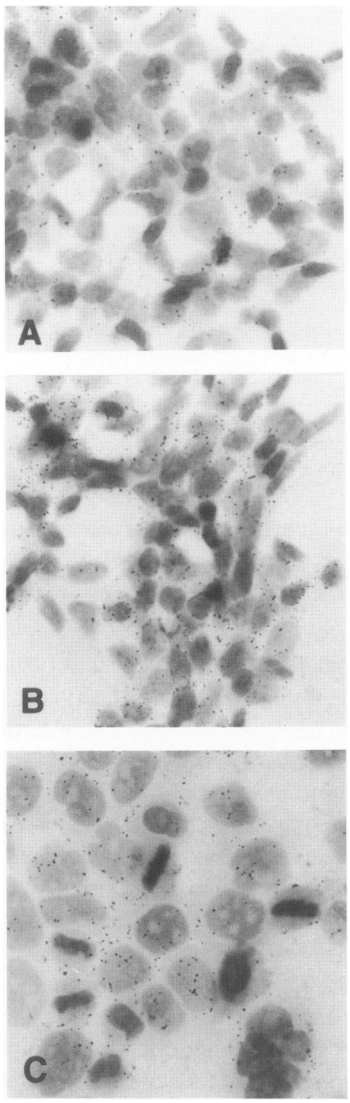


Figure 3. *RNA* in situ *hybridization of* mdr-*1/P-glycoprotein mRNA in SH-SY5Y cells. The cells were plated on microscope slides in tissue culture dishes, and fixed in 4% paraformaldehyde after 48 hours treatment with retinoic acid. Shown in the panels at ×500 magnification are* **(A)** *untreated SH-SY5Y cells;* **(B)** *retinoic acid-treated SH-SY5Y cells; and* **(C)** *KB-8-5 cells, positive control for* mdr-*1/P-glycoprotein expression.*

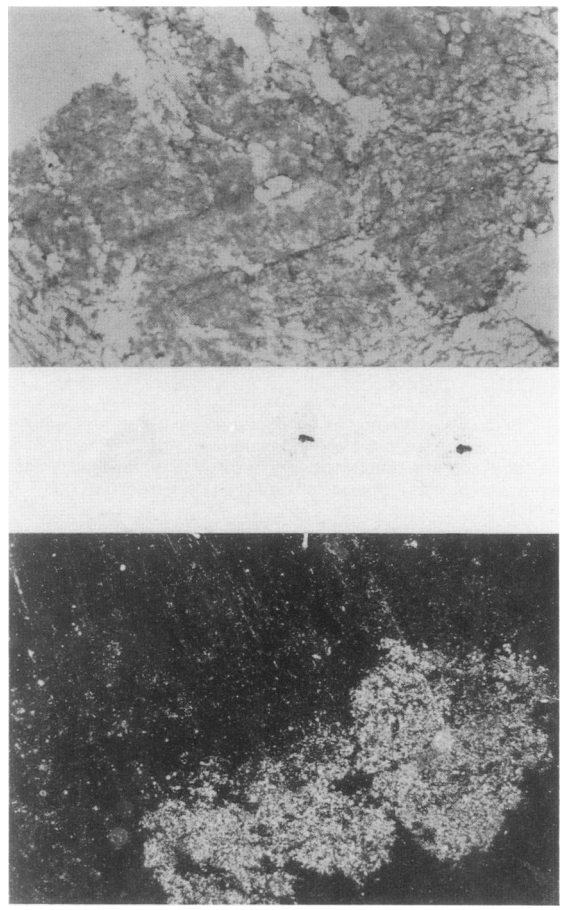
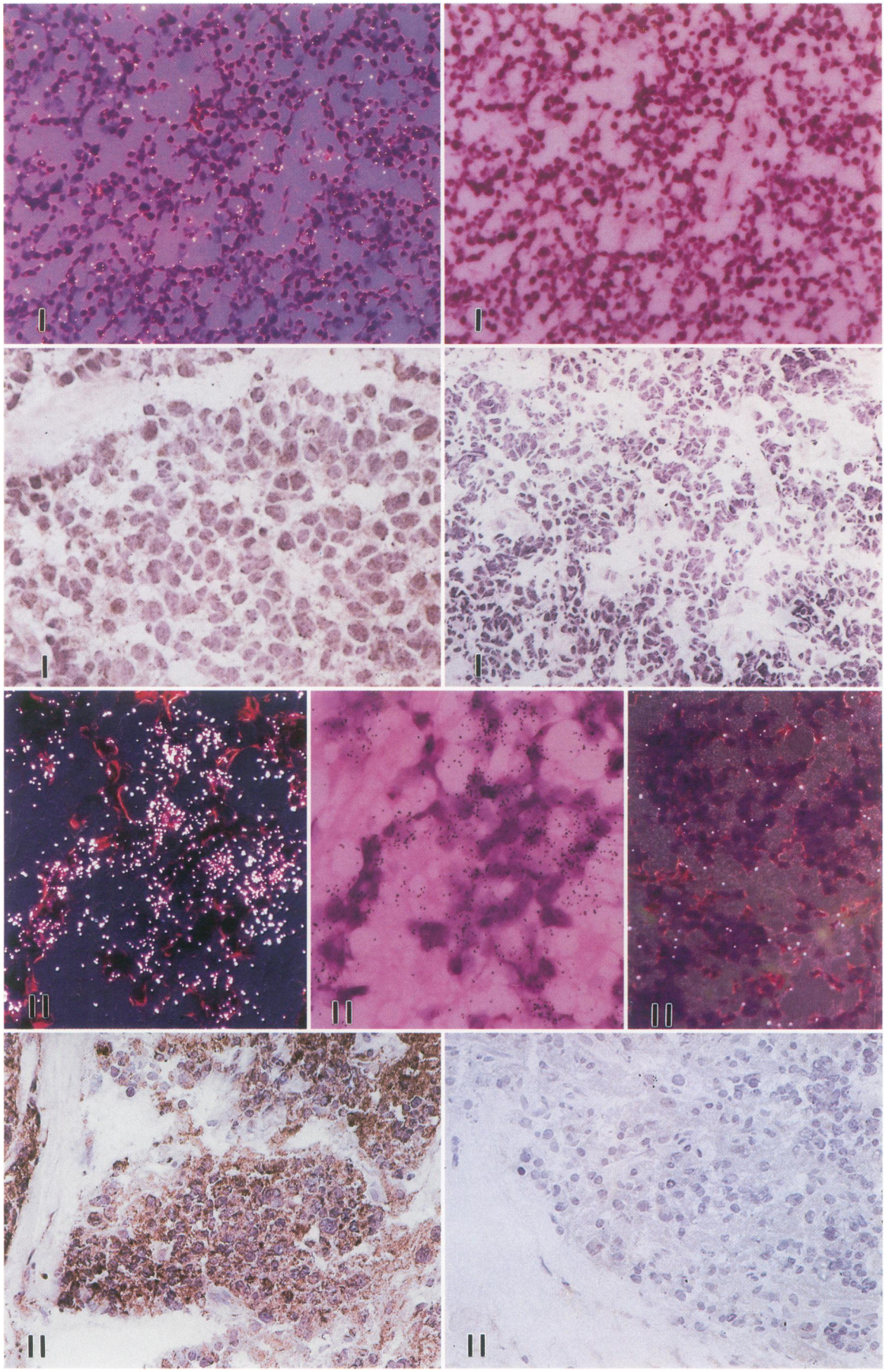


Figure 4. *RNA* in situ *hybridization analysis of a grade II neuroblastoma. Tumor 12-D01 demonstrated a single focus of 4+* mdr-*1 mRNA expression. The figure shows the bright-field photomicrograph at ×63 magnification in the upper panel with no obvious differences in morphology seen at low or high power. In the lower panel, the corresponding dark-field photomicrograph shows the 4+ focus. Autoradiograms of three sections are shown in the inset. The two sections on the right were hybridized with a* mdr-*1 mRNA antisense probe, and the one on the left, the sense control. The signal in the tissue surrounding the focus is low, slightly above control.*

RNA *in situ* hybridization and immunohistochemical analysis of four neuroblastoma tumors representing each stage of differentiation. Each panel shows the RNA *in situ* antisense hybridization, the MRK-16 immunohistochemistry, and the results with the nonspecific control antibody, IgG γ2a for each one of the histologic grades.

Grade I tumors expressed the lowest levels of *mdr*-1/P-glycoprotein seen in our studies, although all of the samples appeared to express some *mdr*-1 mRNA or P-glycoprotein. In tumors of this type, an occasional intensely positive cell could be found in a field of low-level staining cells. The most positive grade II tumor (08-Z01) is presented in Figure 5. Although the tumor was graded as II in our study, the pathologic diagnosis of ganglioneuroblastoma from the surgical specimen suggests that there may have been other areas that demonstrated further

Figure 5. *P-glycoprotein and* mdr-*1 mRNA expression in four neuroblastoma tissues, representing each histologic grade. Each panel contains dark- and bright-field photomicrographs of the RNA* in situ *hybridization, and P-glycoprotein immunostains of each tumor with an associated control antibody stain. For the grade I tumor (11-D02), the P-glycoprotein immunostaining is shown at ×500 magnification; the other panels are ×250. The grade II tumor (08-Z01) is shown at ×500 magnification for* mdr-*1* in situ *hybridization. The sense control (the last photograph on the right) for the specificity of the RNA* in situ *hybridization signal and the immunostaining are shown at ×250. The grade III tumor (09-EE02) is shown at ×250 magnification throughout. For the grade IV tumor (04-N01), P-glycoprotein immunostaining is shown at ×250, whereas RNA* in situ *hybridization is shown at ×500. In the dark-field photographs, the* in situ *signal appears as a bright spot, which in the identical bright field is a black grain. In the immunostains, histologically similar but not identical fields are shown for control sections.*



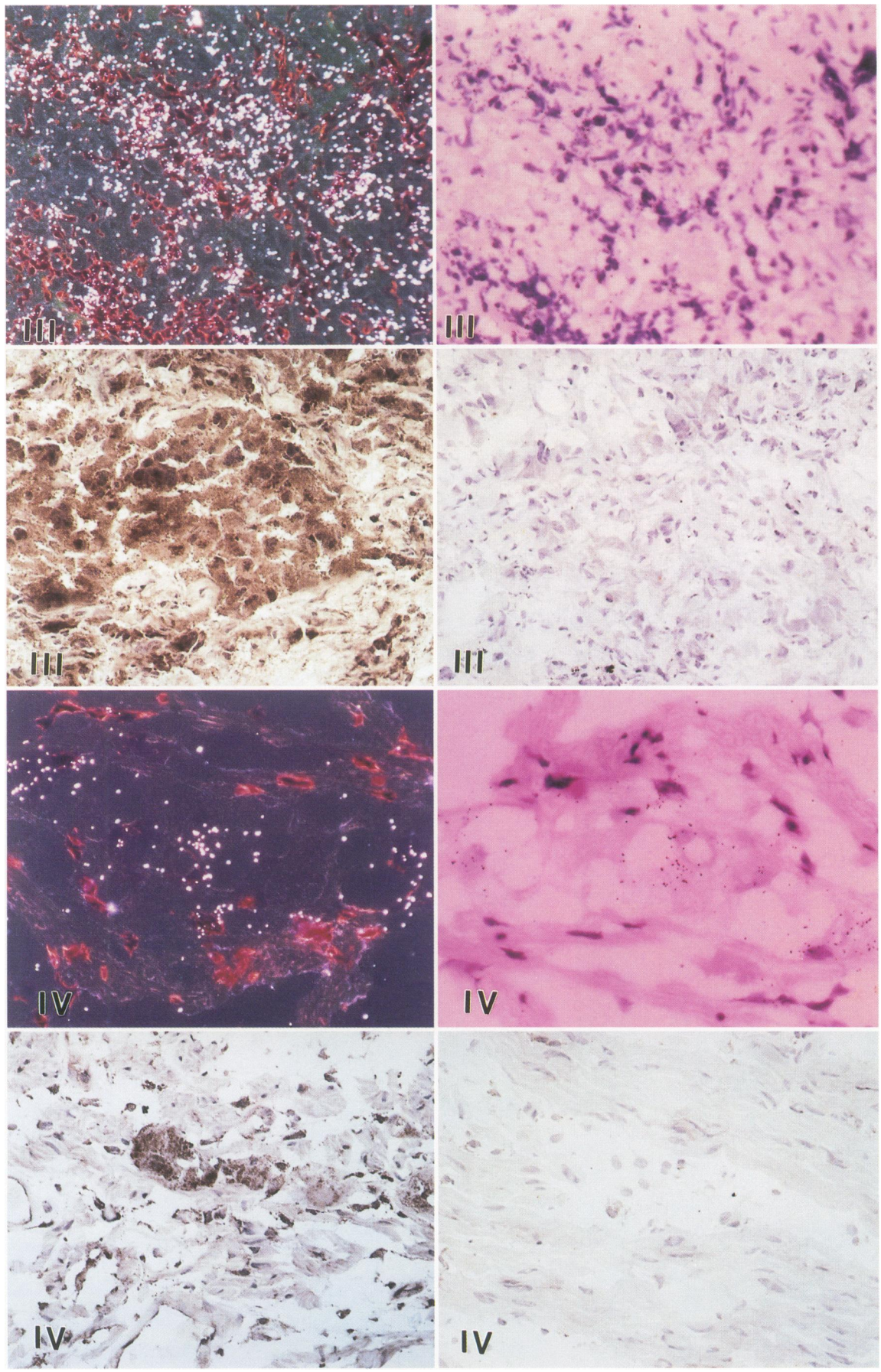

III
III
III
III
IV
IV
IV
IV

Table 3. *Scattergram Showing Expression of* mdr-*1 mRNA and P-glycoprotein in Neuroblastoma Samples*

mdr-1 mRNA†					
	4+		*(*)‡		*‖
	3+			*	
	2+	*			
	0–1+	***	***(*)	*	
	Grade	I	II	III	IV
P-glycoprotein†					
	4+		*		*‖
	3+			*	
	2+	*	**		
	0–1+	***	**	*	
	Grade	I	II	III	IV

† Expression of *mdr*-1 mRNA and P-glycoprotein assigned levels from 1 to 4+ is correlated with grade. Levels of P-glycoprotein and *mdr*-1 mRNA were significantly correlated, with a Pearson's correlation coefficient of 0.873 ($P = 0.0002$). Increased differentiation was significantly correlated with increased P-glycoprotein expression, Pearson's correlation coefficient 0.584 ($P = 0.046$). Increased *mdr*-1 expression and differentiation approached but did not achieve statistical significance ($P = 0.064$).

‡ The grade II tumor in parentheses indicates the focus of 4+ *mdr*-1 mRNA expression in 12-D01 that was found in serial tissue sections, while the rest of the tumor was 1+. The P-glycoprotein sections were distant from this area, and the focus was not seen again.

‖ The 4+ expression in the grade IV ganglioneuroma indicates the level of expression in the ganglionic cells, and not the absence of expression in the stroma.

differentiation. Although it was the initial surgery for the patient, preoperative chemotherapy had been given, with a good response. Ubiquitous *mdr*-1 expression both by RNA *in situ* hybridization and immunohistochemical staining was observed, but the intensity was variable. Both primitive and more differentiated cell types within the section demonstrated expression; and the intensity of expression was not consistently higher in one or the other cellular subpopulation. The grade III tumor demonstrates intense expression of P-glycoprotein as well. Both the primitive and differentiated cell types within the sections demonstrate expression. Last, in the grade IV tumor, expression is located in the large ganglionic cells found between the axonal/Schwannian bundles. These cells were not as well preserved in the RNA *in situ* hybridization, with its more stringent washing conditions, as in the H&E stain or in the immunohistochemistry slides. An occasional large cell remained, however, and demonstrated significant hybridization, as shown in the panel. In one experiment, some of the spindle-shaped cells in the 'stroma'-rich region showed P-glycoprotein staining (data not shown). Both the 'stroma'-rich, poorly differentiated tumor (grade III) and the 'stroma'-rich ganglioneuroma (grade IV) were obtained from patients who had received preoperative chemotherapy. The RNA *in situ* hybridization and immunohistochemical techniques used in these studies are best used as qualitative assays. Subjectively scoring the levels of expression from 1+ to 4+, however, gave consistent results. Table 3 summarizes in scattergram form the findings from all of the tumors.

Discussions

In the present study, we have demonstrated expression of *mdr*-1/P-glycoprotein by RNA *in situ* hybridization and immunohistochemistry in 12 neuroblastoma samples from 11 patients. Expression of *mdr*-1/P-glycoprotein increased with increasing differentiation. The 'stroma'-poor poorly differentiated neuroblastomas had lower levels of *mdr*-1/P-glycoprotein. The intermediate grades had higher levels of expression, although both primitive and more differentiated cells expressed the gene at comparable levels within the tumor. The grades III and IV 'stroma'-rich neuroblastomas demonstrated the highest levels of *mdr*-1 mRNA and P-glycoprotein expression. A very well-differentiated ganglioneuroma demonstrated ample P-glycoprotein expression in the ganglionic cells, but none in the vast stroma, which made up the majority of the tissue. Striking heterogeneity was rarely observed, although in one case a focal area of intense *mdr*-1 mRNA expression was noted against a background of low-level expression. These results extend our previous *in vitro* observations showing increased *mdr*-1 expression in neuroblastoma cell lines after differentiation with retinoic acid.[14] Similar observations were recently reported by Nakagawara et al[34] in 35 cases of neuroblastoma analyzed by RNA slot blot for *mdr*-1 and N-*myc* expression.[34] In these cases, *mdr*-1 and N-*myc* were inversely correlated. Because N-*myc* expression decreases with differentiation *in vitro*,[35–37] the inverse correlation suggested that *mdr*-1 mRNA expression increased with differentiation in these tumors. This suggestion was supported by the finding of higher levels of *mdr*-1 in the histologically more differentiated tumors. As in previous series, N-*myc* amplification correlated with a poor prognosis. By contrast, low *mdr*-1 expression was associated with a worse survival, and high *mdr*-1 with improved survival. In the 11 patients presented here, survival could not be correlated with *mdr*-1 or P-glycoprotein expression, perhaps because of sample size.

Although these observations are not conclusive, and more cases need to be examined, these studies would predict that if the *mdr*-1 gene functions in neuroblastoma as a drug efflux pump, the natural products are ultimately likely to be ineffective drugs in treatment of neuroblastoma. Evidence that the gene functions in human neuroblastoma has not been demonstrated. Cell lines derived from untreated colon and kidney cancers which express P-glycoprotein, however, have increased drug accumulation after addition of P-glycoprotein antagonists *in vitro* (unpublished data). Unless alterations occur in tissue culture, the inference can be made that presence of the protein *in vivo* indicates an active drug efflux pump. It must be noted, however, that when retinoic acid was added to the SK-N-SH neuroblastoma cell line and its

subline, SH-SY5Y, P-glycoprotein expression increased without a decrease in drug accumulation. The data suggested a nonfunctional P-glycoprotein in the retinoic-acid–treated cells. Selection of these cell lines with natural products has resulted in multidrug resistance with increased P-glycoprotein.[19] Thus it remains unclear whether the failure of P-glycoprotein to function as a drug efflux pump after differentiation is inherent within the unselected neuroblastoma cell lines, or a consequence of the differentiating agent treatment. The finding of Nakagawara that higher levels of *mdr*-1 were associated with a better prognosis and with a differentiated histology could be consistent with a nonfunctional drug efflux pump.[34] Determination of the functionality of the *mdr*-1 gene in the tumors of patients will require prospective clinical trials using *mdr*-1 reversing agents, or the development of agents suitable for assessing drug accumulation *in vivo*.

It is interesting that the tumors with the highest levels of P-glycoprotein were exposed to chemotherapy. Responses were reported in all three patients. Increased *mdr*-1 mRNA expression has been reported after chemotherapy in neuroblastoma.[38] Early reports in neuroblastoma therapy suggested that increased differentiation could be observed after treatment in some patients. This differentiation after chemotherapy could then be associated with increased *mdr*-1 expression. Because the patients were responding, it is also possible that the high levels of *mdr*-1/P-glycoprotein expression were a result of the survival of *mdr*-1/P-glycoprotein-expressing subpopulations that were more resistant to treatment.

We thus have described correlation of *mdr*-1/P-glycoprotein expression with differentiation in human neuroblastoma. These findings extend the *in vitro* results that demonstrate that retinoic acid increases *mdr*-1/P-glycoprotein expression in neuroblastoma cell lines. The central question arising from these findings is what role does P-glycoprotein play in clinical resistance in neuroblastoma if it is expressed in 'good prognosis' tumors? Perhaps the tumors are good prognosis because of an underlying biologically less aggressive, more differentiated phenotype rather than because they are more responsive to chemotherapy. Alternatively if induction of a nonfunctional P-glycoprotein in neuroblastoma in culture after retinoic acid is indicative of lack of function of the P-glycoprotein in these differentiated tumors, then perhaps drug resistance requires yet another alteration to achieve a P-glycoprotein that can transport drugs. Understanding the answers to such questions could provide potentially new treatment areas for neuroblastoma, perhaps including differentiating agents at one point in therapy, or P-glycoprotein antagonists at another.

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