Jpn. J. Cancer Res. **89**, 1276–1283, December 1998

Expression of MRP and cMOAT in Childhood Neuroblastomas and Malignant Liver Tumors and Its Relevance to Clinical Behavior

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Advanced neuroblastoma and malignant liver tumor are representative childhood cancers for which combined chemotherapy including cisplatin and doxorubicin is routinely performed. The prognosis of patients with tumors which develop multiple drug resistance (MDR) is unfavorable. To elucidate the role of multidrug resistance-associated protein (MRP) and canalicular multispecific organic anion transporter (cMOAT) in the clinical behavior of the tumors, we examined 42 neuroblastomas and 10 malignant liver tumors for the expressions of MRP and cMOAT by quantitative RNA-polymerase chain reaction (PCR). The amplification and expression of N-*myc* oncogene in the neuroblastomas were also investigated. We found a close association between MRP and N-*myc* expression in each neuroblastoma sample but no significant relationship between MRP expression and the patients' outcome. The forced expression of N-*myc* failed to enhance the expression of MRP in N-*myc* transfected neuroblastoma cell lines. cMOAT was rarely expressed in the neuroblastomas, but was frequently expressed in the malignant liver tumors. The expression of MRP and cMOAT in the childhood liver tumors was more common and higher, especially in advanced cases with a poor outcome, than that observed in normal liver or in 9 hepatocellular carcinomas from adult patients. The enhanced expression of these genes might be characteristic of childhood malignant liver tumors and related to their clinical chemoresistance.

Key words: MRP — cMOAT — Childhood malignant liver tumor — Neuroblastoma — N-*myc*

The acquisition of multidrug resistance (MDR) by tumor cells is frequently observed after the administration of a single cytostatic drug, and is a major obstacle to chemotherapy in patients with cancer. Because childhood cancers generally respond well to anticancer drugs, chemotherapy plays an important role in multimodal treatment for them. Neuroblastoma and malignant liver tumor (hepatoblastoma and hepatocellular carcinoma, adult type) are representative childhood cancers which require intensive chemotherapy, including cisplatin and doxorubicin, to be cured. However, advanced tumors often display MDR, which results in a fatal outcome. Information about the MDR phenotype of an individual tumor would contribute to decisions about appropriate treatment strategies.

One of the mechanisms underlying MDR is an outward efflux of chemotherapeutic agents by membrane-bound P-glycoprotein, encoded by the *MDR1* gene.[1)] Although MDR1 expression in relation to the MDR phenotype in certain cancers is well-characterized, evidence concerning the relation of MDR1 expression to the clinical behavior of neuroblastomas is contradictory.[2–4)] Multidrug resistance-associated protein (MRP) is another membrane transport protein, the overexpression of which has been proposed to be associated with the non-P-glycoprotein-mediated MDR phenotype *in vitro* and *in vivo*.[5–8)] In analyses of MRP expression in neuroblastomas, the enhanced expression of MRP was reported to correlate with the amplification and overexpression of N-*myc* gene and a poor outcome of the patients.[9, 10)] However, MRP has not been shown to mediate resistance to cisplatin, which is the most powerful anticancer drug for neuroblastomas.[8)] Further investigations are necessary to elucidate the role of MRP in the clinical drug resistance of neuroblastomas.

The human canalicular multispecific organic anion transporter (*cMOAT*) gene was recently cloned from a cisplatin-resistant human head and neck cancer cell line, by targeting the conserved ATP-binding domain in MDR1 and MRP.[11)] Human cMOAT was expressed at enhanced levels in the liver and a subset of cisplatin-resistant cell lines,[12)] and the introduction of antisense cDNA of cMOAT enhanced the sensitivity of multiple anticancer drugs, including cisplatin, in human hepatic cancer cells.[13)] However, analyses of cMOAT expression in primary cancers and its relevance to clinical behavior have not been documented.[13)]

In the present study, to clarify the clinical significance of MRP and cMOAT expression in childhood cancers, we analyzed their expression in 42 neuroblastomas and 10

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malignant liver tumors by quantitative RNA-polymerase chain reaction (PCR).[14, 15] Nine hepatocellular carcinomas from adult patients were also analyzed for comparison with the childhood liver tumors. We also examined neuroblastomas for expression of N-*myc* oncogene, the amplification of which is a powerful predictor of a poor outcome,[16] and analyzed MRP expression in N-*myc*-transfected neuroblastoma cell lines to determine the effect of N-*myc* overexpression on MRP expression.

MATERIALS AND METHODS

Patients and tumor specimens Forty-two patients with neuroblastoma were treated at Chiba University Hospital, Chiba Children's Hospital or Matsudo Municipal Hospital between 1987 and 1997. The median follow-up period after diagnosis for the surviving children was 78 months (range, 17 to 131; disease-free survival). Of the 42 cases, 20 were metastatic disease and the remaining 22 were localized disease. The neuroblastoma tissues were obtained by biopsy or surgery prior to chemotherapy. After the biopsy, all patients with an unresectable tumor received intensive chemotherapy including cisplatin, doxorubicin and cyclophosphamide. Genomic amplification of N-*myc* (more than 10 copies per haploid set) was found in 14 tumors, all of which were metastatic disease. A fatal outcome was observed in 8 of the 14 patients with the N-*myc*-amplified tumor and in 3 of the 28 patients with non N-*myc*-amplified tumor. All 11 children with a fatal outcome died of disease resulting from chemoresistance.

Nine children with a malignant liver tumor were treated at Chiba University Hospital between 1986 and 1996. Eight cases were hepatoblastomas and one case was hepatocellular carcinoma, adult type. The hepatoblastoma tissues were obtained by surgery before (2 cases) or after (6 cases) chemotherapy. Two specimens were obtained from a child with hepatocellular carcinoma before and after chemotherapy. Five of the 9 children with malignant liver tumor have remained alive without disease for over 2 years (24 to 135 months), and the remaining 4 patients died of disease without responding to the combined chemotherapy, including cisplatin and doxorubicin.

Normal tissues and cell lines RNA from normal tissues (kidney, spleen, liver and lung) and 10 neuroblastoma cell lines (IMR32, RT-BM-1, SK-N-SH, NB69, NB69N, NB69S, NB-1, GOTO, cNBI and LA-N-5) was used as the reference standard for the expression of MRP or cMOAT (normal tissues) and N-*myc* (neuroblastoma cell lines), respectively. SK-N-SH, IMR32, GOTO and NB-1 were obtained from the Japanese Cancer Research Resources Bank. LA-N-5 was kindly provided by Dr. Robert C. Seeger (Children's Hospital of Los Angeles, Los Angeles, CA). RT-BM-1[17] was kindly provided by Dr. Tohru Sugimoto (Miyazaki Medical College, Miyazaki). NB69N and NB69S are clonal sublines of NB69, and cNBI was described previously.[18] The terminal differentiation of RT-BM-1 cells was induced by *trans*-retinoic acid (Sigma Chemicals, St. Louis, MO), as described previously.[18]

RNA PCR Total cytoplasmic RNA (5 μg) was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase and random hexanucleotide primers, essentially as described previously.[18] Target (MRP, cMOAT or N-*myc*) and control β2-microglobulin gene-sequences were coamplified in the same reaction, using the following gene-specific oligonucleotide primers:

5′-TCTGGGACTGGAATGTCACG-3′
(MRP, forward);
5′-CAGGAATATGCCCCGACTTC-3′
(MRP, reverse);
5′-CTGCCTCTTCAGAATCTTAG-3′
(cMOAT, forward);
5′-ACCAGCCTCTGTAACTTCGT-3′
(cMOAT, reverse);
5′-GACCACAAGGCCCTCAGTAC-3′
(N-*myc*, forward);
5′-GTGGATGGGAAGGCATCGTT-3′
(N-*myc*, reverse);
5′-ACCCCCACTGAAAAAGATGA-3′
(β2-microglobulin, forward);
5′-ATCTTCAAACCTCCATGATG-3′
(β2-microglobulin, reverse).

The expected sizes of the PCR products when using these sets of primers are 262 (MRP), 267 (cMOAT), 240 (N-*myc*) and 120 (β2-microglobulin) base pairs. Aliquots of cDNA corresponding to 50 ng of RNA were subjected to PCR in a final volume of 25 μl using 1 unit of AmpliTaq Gold Polymerase (Perkin Elmer Cetus, Norwalk, CT). An initial denaturation of 9 min at 94°C was followed by 36 (for MRP and cMOAT) or 32 (for N-*myc*) cycles of a 30 s denaturing step at 94°C, a 30 s annealing step at 57°C and a 30 s extension step at 72°C. These PCR conditions were determined on the basis of preliminary experiments using the normal tissues and 10 neuroblastoma cell lines, which indicated that the PCR products of the target and control genes were amplified in parallel with the number of PCR cycles within the range from 33 to 39 cycles (for MRP and cMOAT) or 28 to 36 cycles (for N-*myc*); three independent PCR studies resulted in almost identical levels for all target and control genes (data not shown). Following the PCR, 8 μl of PCR reaction mixture was subjected to electrophoresis on 2.5% agarose gels.

Estimation of gene expression The PCR products in gels containing a DNA staining solution, SYBER Green, were visualized by ultraviolet transillumination and recorded as digital images by a Kodak Digital Science DC40 camera, and the intensity of each band was mea-

sured by the 1D Image Analysis Application program (Eastman Kodak, Rochester, NY).

N-*myc* transfection Whole human N-*myc* cloned in EMBL3[19] was digested with *Xba*I and *Hin*cII, and a 4.7 kb fragment of N-*myc* including the entire coding region (from the middle of intron 1 to the middle of exon 3)[20] was subcloned into pTV119N. After the addition of *Xba*I linker at the *Hin*cII site, the *Xba*I–*Xba*I fragment of the 4.7 kb N-*myc* gene was cloned into an expression vector, pRC/RSV (Invitrogen, San Diego, CA) containing a Rous sarcoma virus long terminal repeat and bovine growth hormone poly A signal. Then 5×10^6 neuroblastoma cells (NB69N) were electroporated with 10 μg of the N-*myc* expression vector, and seven stable N-*myc* transfectants were selected in 400 μg/ml of G418 one day after the transfection. The control transfectant, 69NR, was established by introducing the vector not containing the N-*myc* gene. The introduction of the N-*myc* gene, the exogenous expression of the N-*myc* mRNA and the nuclear localization of the N-*myc* protein, not in 69NR, but in the N-*myc* transfectants were confirmed by Southern blot hybridization, northern blot hybridization and immunofluorescence using an anti-N-*myc* antibody (Cambridge Research Biochemicals, Valley Stream, NY), respectively.

Statistical analysis The Mann-Whitney *U*-test was used to evaluate the significance of the gene expression in relation to spread of the disease, prognosis of the patients and N-*myc* gene amplification. The probability of survival of the patients was calculated by the product limit method of Kaplan and Meier and results were compared using the log rank test.

RESULTS

The expressions of MRP, cMOAT and N-*myc* in normal tissues, 10 neuroblastoma cell lines, 42 primary neuroblastomas, 10 primary childhood malignant liver tumors and 9 hepatocellular carcinomas from adult patients were analyzed by quantitative RNA-PCR. Representative results are shown in Fig. 1. The mean PCR ratios of the target genes (MRP, cMOAT and N-*myc*) to control β2-microglobulin in the normal tissues and cancers are summarized in Table I.

MRP expression was low in the liver, moderate in the kidney and spleen, and relatively high in the lung. cMOAT expression was very low in the kidney, high in the liver and not detectable in the spleen and lung. These expression patterns corresponded to previously reported observations.[11, 21, 22] All 10 neuroblastoma cell lines expressed MRP, the levels of which were higher than those observed in the lung. In contrast, cMOAT expression was found only in IMR32, GOTO, NB69 and its sublines, and its expression levels were lower than those found in the kidney. High levels of N-*myc* expression were restricted to the N-*myc*-amplified neuroblastoma cell lines (IMR32, RT-BM-1, NB-1, GOTO, cNBI and LA-N-5), and very low or undetectable N-*myc* expression was found in the non-N-*myc*-amplified neuroblastoma cell lines (SK-N-SH, NB69, NB69N and NB69S) and in the normal tissues, as reported previously.[18, 23–25]

In the analyses of the 42 primary neuroblastoma specimens, the expression of MRP and N-*myc* was observed in

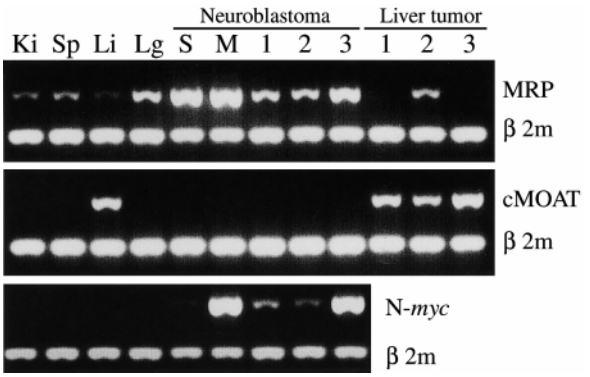

Fig. 1. Representative results of competitive RNA-PCR analyses for the expression of the target (MRP, cMOAT and N-*myc*) and control (β2-microglobulin) genes. Shown are 4 normal tissues, 2 neuroblastoma cell lines and 3 tumors from patients with neuroblastoma and malignant liver tumor. The PCR products were separated on 2.5% agarose gels. The PCR product sizes are 262 (MRP), 267 (cMOAT), 240 (N-*myc*) and 120 (β2-microglobulin) base pairs. Ki, kidney; Sp, spleen; Li, liver; Lg, lung; S, SK-N-SH; M, IMR32; neuroblastoma 1, localized disease; 2, metastatic disease without N-*myc* amplification; 3, metastatic disease with N-*myc* amplification; liver tumor 1, resectable hepatoblastoma; 2, unresectable hepatoblastoma; 3, metastatic hepatocellular carcinoma, adult type.

Table I. Mean PCR Ratios of MRP, cMOAT and N-*myc* in Normal Tissues and Cancers

	MRP	cMOAT	N-*myc*
kindney (3 experiments)	0.14±0.02	0.05±0.01	—
spleen (3 experiments)	0.17±0.02	—	—
liver (3 experiments)	0.07±0.01	0.45±0.02	—
lung (3 experiments)	0.38±0.05	—	—
neuroblastoma cell lines (n=10)	1.21±0.08	—	1.60±0.43
primary neuroblastomas (n=42)	0.32±0.04	—	0.65±0.12
childhood malignant liver tumors (n=10)	0.10±0.04	0.40±0.10	ND
hepatocellular carcinomas from adult patients (n=9)	—	0.08±0.04	ND

—, non-measurable levels; ND, not done.

39 and 39 tumors, respectively. The expression of cMOAT at detectable levels was found in only 2 cases, in which the level was as low as that observed in the kidney. The difference of the mean PCR ratios in subgroups categorized by clinical and biological features (spread of the disease, N-*myc* amplification and prognosis of the patients) is shown in Fig. 2. The metastatic tumors expressed higher levels of MRP and N-*myc* than the localized tumors. However, a significant difference was found only in the relation between N-*myc* expression levels and the spread of the disease (P=0.2125 and 0.0048 for MRP and N-*myc*, respectively). Significantly higher expressions of MRP and N-*myc* were observed in N-*myc*-amplified tumors compared to the tumors without N-*myc* amplification (P=0.0117 and P<0.0001 for MRP and N-*myc*, respectively). In contrast, neither MRP nor N-*myc* expression was significantly associated with the disease-free survival of the patients, although both genes showed a tendency to be more highly expressed in the unfavorable group (P=0.5480 and 0.1608 for MRP and N-*myc*, respectively). When the levels of expression of MRP and N-*myc* in an individual tumor were classified as high or low in relation to the mean PCR ratio calculated for all tumors, the cumulative disease-free survival data obtained by the method of Kaplan and Meier indicated better disease-free survival of the patients with tumors expressing low levels of MRP or N-*myc*. However, this association between the expression of MRP or N-*myc* and patient prognosis was not significant (χ^2=0.211, P=0.6458 and χ^2=2.085, P=0.1487 for MRP and N-*myc*, respectively, data not shown).

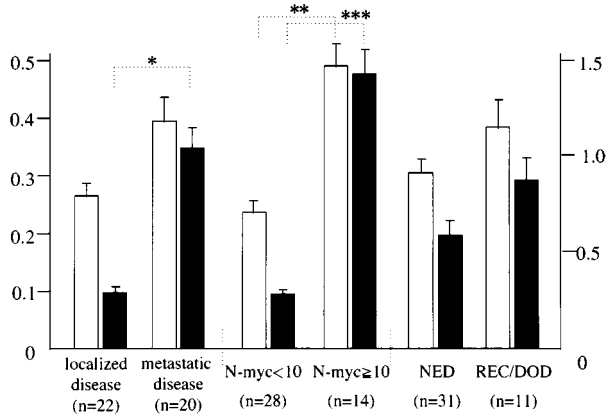

Fig. 2. Mean PCR ratios of target genes (MRP and N-*myc*) to control β2-microglobulin gene in subgroups categorized by clinical and biological features (spread of the disease, N-*myc* amplification and prognosis of the patients). NED, no evidence of disease; REC/DOD, recurrence or death of disease; *, P=0.0048; **, P=0.0117; ***, P<0.0001. □ MRP (left scale), ■ N-*myc* (right scale).

The relation of the PCR ratio of MRP and N-*myc* expression for each sample and its relevance to the disease-free survival of the patients is shown in Fig. 3. The results revealed a highly significant correlation between the MRP and N-*myc* expression (r=0.795, P<0.0001), and no involvement of the expression of these genes in the prognosis of the patient.

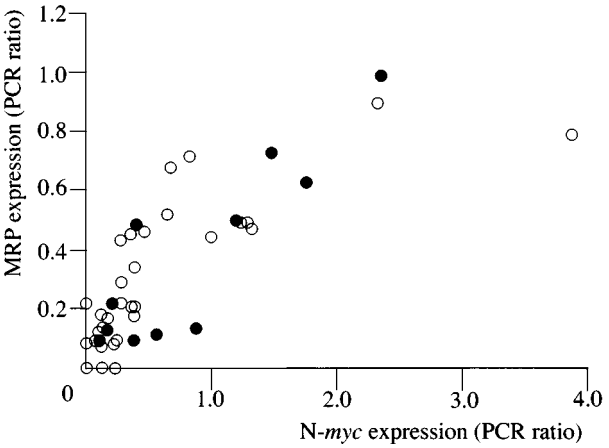

Fig. 3. Relation of the PCR ratios of the MRP and N-*myc* expressions for each neuroblastoma and its relevance to prognosis, indicating a highly significant correlation between the MRP and N-*myc* expressions (r=0.795, P<0.0001), and no involvement of the expression of these genes at high levels in a fatal outcome. ● recurrence or death of disease, ○ no evidence of disease.

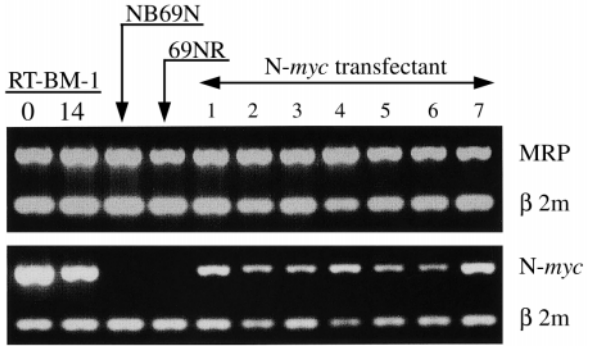

Fig. 4. Expressions of N-*myc* and MRP in parental NB69N, control 69NR, 7 N-*myc* transfectants, RT-BM-1 (0) and its chemically differentiated cells treated with retinoic acid for 14 days (14). The differentiated RT-BM-1 cells expressed decreased levels of N-*myc*, and significant levels of N-*myc* were observed in the 7 N-*myc* transfectants, but not in NB69N or 69NR. There was no obvious change in the levels of MRP expression in these cell lines.

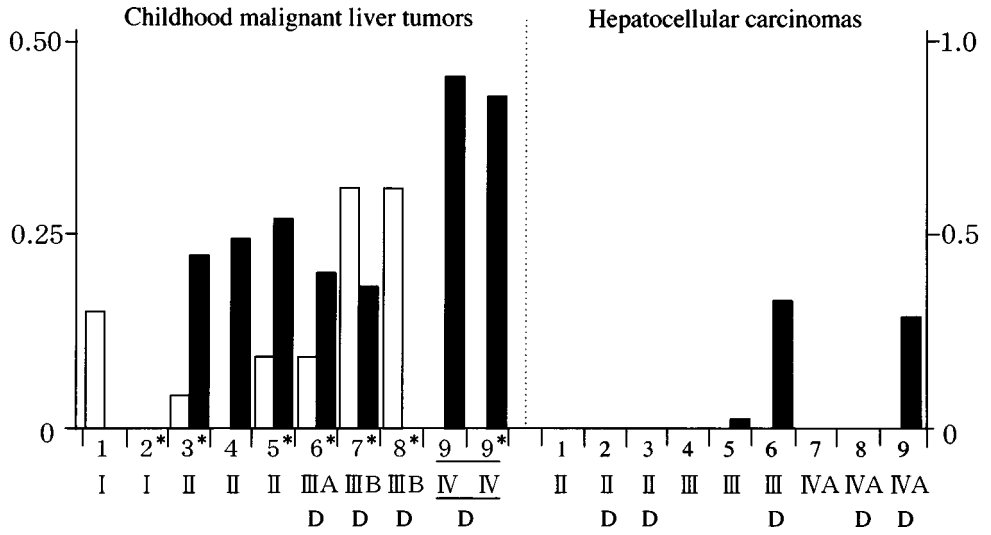

Fig. 5. PCR ratios of target genes (MRP and cMOAT) to control β2-microglobulin gene in 10 malignant liver tumors from 9 children, and 9 hepatocellular carcinomas from adult patients. The pathology of the childhood malignant liver tumor from cases 1 to 8 was hepatoblastoma, and that of case 9 was hepatocellular carcinoma, adult type. The tumors were staged according to the spread of the disease (the classification of the Japanese Society of Pediatric Surgeons and Liver Cancer Study Group of Japan). D, death of disease (other patients are alive without tumor). *, chemotherapy prior gene analyses. □ MRP (left scale), ■ cMOAT (right scale).

To determine the direct effect of the N-*myc* oncogene on the regulation of the transcription of MRP expression, we transfected the N-*myc* expression vector into the control vector into the neuroblastoma cell line NB69N, originally harboring a single copy of N-*myc*, and established 7 stable N-*myc* transfectants and a control transfectant, 69NR. Compared with the parental cell line NB69N and the control 69NR, the N-*myc* transfectants did not differ in morphological appearance or growth in normal medium, but showed about 6-fold more efficient of colony formation in soft agar (data not shown). Fig. 4 shows the expressions of N-*myc* and MRP in NB69N, 69NR, the 7 N-*myc* transfectants, RT-BM-1 and its chemically differentiated cells following incubation with retinoic acid for 14 days. The differentiated RT-BM-1 cells expressed decreased levels of N-*myc*. While NB69N and 69NR expressed undetectable levels of N-*myc*, significant levels of N-*myc* were observed in all 7 N-*myc* transfectants. However, there was no obvious change in the levels of MRP expression in these cell lines.

In the analyses of the 10 primary childhood malignant liver tumors from 9 children, expressions of MRP and cMOAT were observed in 6 and 7 tumors, respectively (Fig. 5). No relationship between the gene expressions and the degree of histological maturation or serum α-fetoprotein levels was found (data not shown). However, 3 of the 4 tumors with a fatal outcome expressed either MRP or cMOAT at high levels. The expression of MRP and cMOAT in the 9 hepatocellular carcinomas from adult patients was also examined, because the highest expression of cMOAT was observed in a childhood hepatocellular carcinoma. cMOAT expression was found in only 3 of these tumors, and the levels were below those observed in the normal liver and childhood liver tumors. None of the 9 hepatocellular carcinomas from adult patients expressed MRP at a measurable level (Fig. 5).

DISCUSSION

Neuroblastoma is the most common malignant solid tumor in early childhood. The metastatic status of neuroblastoma warrants the administration of megadoses of anticancer drugs, because metastatic tumors generally have lost the ability to undergo neuronal differentiation, as might be expected from the frequent amplification of N-*myc* and decreased expression of genes related to neuronal differentiation.[15, 16, 26–28] However, there is a divergence of long-term prognoses among the patients with metastatic neuroblastoma, even if the biological phenotype of their tumors is almost identical.[15] Since a major cause of a fatal outcome is MDR, analysis of the expression of genes related to the MDR phenotype in neuroblastomas is clinically important.

Our present study of 42 neuroblastomas indicated that the expression of MRP was highly correlated with the expression of N-*myc*, but not with the spread of disease or

the outcome. A close relationship between N-*myc* and MRP expressions was also reported by Bordow *et al*.[9] The overexpression of N-*myc* was restricted to N-*myc*-amplified tumors, but N-*myc*-amplified tumors did not always express N-*myc* at enhanced levels in the present study. This finding concurs with previous observations of ours[18, 29] and others,[24] and these phenomena reduce the prognostic value of N-*myc* expression for patients with neuroblastomas.[25] In fact, the present study showed that N-*myc* expression at high levels was related to metastatic disease, but not significantly related to an unfavorable outcome. Consequently, the levels of MRP expression also did not show a close association with the clinical outcome. However, this conclusion does not agree with that by Norris *et al*.[10] They concluded that a high expression of MRP was the most powerful indicator of a poor outcome for patients with neuroblastoma, and suggested the possibility that transcription of MRP is regulated through E-box motifs, the binding site of *myc* family proteins, in the promoter region of MRP by N-*myc* protein.

Our present results, however, failed to demonstrate an up-regulation of MRP expression in N-*myc*-transfected neuroblastoma cells or a down-regulation of MRP expression in N-*myc*-decreased neuroblastoma cells differentiated by retinoic acid. It is not known whether some unidentified gene(s) or mechanism(s) exists that activate(s) expressions of N-*myc* and MRP independently during the carcinogenesis of neuroblastoma. In view of our observation that MRP expression was highly related to N-*myc* expression, it is unlikely that MRP expression has reliable prognostic value for patients with neuroblastoma. In addition, cisplatin, the most effective agent for neuroblastomas, has not been proven to be a substrate for MRP. The role of MRP in the clinical chemoresistance of neuroblastoma thus remains uncertain.

Taniguchi *et al*. recently isolated human cMOAT cDNA from a cisplatin-resistant human cancer cell line.[11] The enhanced expression of cMOAT was suggested to be involved in the resistance to cisplatin and other chemotherapeutic drugs *in vitro*.[12, 13] In the present study, we examined the expression of cMOAT in childhood solid cancers to clarify its relevance to clinical chemoresistance. We found that cMOAT was not expressed in most of the neuroblastomas, but was frequently expressed in childhood malignant liver tumors.

Malignant liver tumor is another commonly observed type of malignant solid neoplasm in early childhood. The prognosis of patients with unresectable tumor or metastatic disease had been unpromising until combined chemotherapy including cisplatin and doxorubicin was introduced as a treatment.[30] However, the clinical course of advanced disease shows a diversity from complete response to progressive disease, even though the identical regimen is applied. This clinical observation raises the possibility of the involvement of MDR in the heterogeneous evolution of the liver tumors, as well as neuroblastomas.

The analyses of the 10 malignant liver tumors revealed that MRP and cMOAT were expressed in 6 and 7 tumors, respectively. Although the mean levels of MRP in the liver tumors were lower than those found in the neuroblastomas, 2 tumors with a fatal outcome expressed relatively higher levels of MRP. In contrast, the levels of cMOAT expression in the liver tumors were comparable to or greater than those expressed in the normal liver tissue. The most abundant expression of cMOAT was observed in the metastatic tumor (hepatocellular carcinoma, adult type) with a fatal outcome. This expression pattern of MRP at low but detectable levels and cMOAT at high levels in the liver tumors resembles that in the normal liver, from which malignant transformation could occur. However, the hepatocellular carcinomas from adult patients expressed no MRP and showed low levels of cMOAT with a low frequency. The expression of MRP and cMOAT at significant levels might be characteristic of childhood malignant liver tumors and confer an MDR phenotype. Analyses of larger numbers of the tumors may resolve this question.

In conclusion, MRP expressed in neuroblastomas is highly related to N-*myc* expression but not to the clinical course of the disease. It seems likely that co-activation of MRP and N-*myc* does not occur by the direct association of N-*myc* protein with the regulatory domain of MRP, as shown by the present N-*myc* transfection assays. We found that childhood malignant liver tumors in advanced stages expressed MRP and cMOAT at higher levels and more frequently than those found in the normal liver and hepatocellular carcinomas from adult patients, indicating that these genes might be involved in the clinical chemoresistance.

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, and a Research Grant from the Ministry of Health and Welfare of Japan. The authors thank Dr. T. Sugimoto and Dr. K. Kawamura for providing the NB cell line RT-BM-1 and the neuroblastoma tumor specimen, respectively.

(Received July 15, 1998/Revised September 7, 1998/Accepted September 10, 1998)

REFERENCES

1) Germann, U. A. P-glycoprotein : a mediator of multidrug resistance in tumour cells. *Eur. J. Cancer*, **32A**, 927–944 (1996).
2) Bourhis, J., Benard, J., Hartmann, O., Boccon-Gibod, L., Lemerle, J. and Riou, G. Correlation of MDR1 gene expression with chemotherapy in neuroblastoma. *J. Natl. Cancer Inst.*, **81**, 1401–1405 (1989).
3) Nakagawara, A., Kadomatsu, K., Sato, S., Kohno, K., Takano, H., Akazawa, K., Nose, Y. and Kuwano, M. Inverse correlation between expression of multidrug resistance gene and N-*myc* oncogene in human neuroblastomas. *Cancer Res.*, **50**, 3043–3047 (1990).
4) Chan, H. S. L., Haddad, G., Thorner, P. S., DeBoer, G., Lin, Y. P., Ondrusek, N., Yeger, H. and Ling, V. P-Glycoprotein expression as a predictor of the outcome of therapy for neuroblastoma. *N. Engl. J. Med.*, **325**, 1608–1614 (1991).
5) Cole, S. P. C., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. U., Duncan, A. M. V. and Deeley, R. G. Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science*, **258**, 1650–1654 (1992).
6) Loe, D. W., Deeley, R. G. and Cole, S. P. C. Biology of the multidrug resistance-associated protein, MRP. *Eur. J. Cancer*, **32A**, 945–957 (1996).
7) Nooter, K., Westerman, A. M., Flens, M. J., Zaman, J. R., Scheper, R. J., van Wingerden, K. E., Burger, H., Oostrum, R., Boersma, T., Sonneveld, P., Gratama, J. W., Kok, T., Eggermont, A. M. M., Bosman, F. T. and Stoter, G. Expression of the multidrug resistance-associated protein (MRP) gene in human cancers. *Clin. Cancer Res.*, **1**, 1301–1310 (1995).
8) Grant, C. E., Valdimarsson, G., Hipfner, D. R., Almquist, K. C., Cole, S. P. C. and Deeley, R. G. Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. *Cancer Res.*, **54**, 357–361 (1994).
9) Bordow, S. B., Haber, M., Madafiglio, J., Cheung, B., Marshall, G. M. and Norris, M. D. Expression of the multidrug resistance-associated protein (MRP) gene correlates with amplification and overexpression of the N-*myc* oncogene in childhood neuroblastoma. *Cancer Res.*, **54**, 5036–5049 (1994).
10) Norris, M. D., Bordow, S. B., Marshall, G. M., Haber, P. S., Cohn, S. L. and Haber, M. Expression of the gene for multidrug-resistance-associated protein and outcome in patients with neuroblastoma. *N. Engl. J. Med.*, **334**, 231–238 (1996).
11) Taniguchi, K., Wada, M., Kohno, K., Nakamura, T., Kawabe, T., Kawakami, M., Kagotani, K., Okumura, K., Akiyama, S. and Kuwano, M. A human canalicular multispecific organic anion transporter (cMOAT) gene is overexpressed in cisplatin-resistant human cancer cell lines with decreased drug accumulation. *Cancer Res.*, **56**, 4124–4129 (1996).
12) Koike, K., Kawabe, T., Tanaka, T., Toh, S., Uchiumi, T., Wada, M., Akiyama, S., Ono, M. and Kuwano, M. A canalicular multispecific organic anion transporter (cMOAT) antisense cDNA enhances drug sensitivity in human hepatic cancer cells. *Cancer Res.*, **57**, 5475–5479 (1997).
13) Narasaki, F., Oka, M., Nakano, R., Ikeda, K., Fukuda, M., Nakamura, T., Soda, H., Nakagawa, M., Kuwano, M. and Kohno, S. Human canalicular multispecific organic anion transporter (cMOAT) is expressed in human lung, gastric, and colorectal cancer cells. *Biochem. Biophys. Res. Commun.*, **240**, 606–611 (1997).
14) Noonan, K. E., Beck, C., Holmayer, T. A., Chin, J. E., Wunder, J. S., Andrulis, I. L., Gazdar, A. F., Willman, C. L., Griffith, B., Von Hoff, D. D. and Roninson, I. B. Quantitative analysis of MDR1 (multidrug resistance) gene expression in human tumors by polymerase chain reaction. *Proc. Natl. Acad. Sci. USA*, **87**, 7160–7164 (1990).
15) Matsunaga, T., Shirasawa, H., Enomoto, H., Yoshida, H., Iwai, J., Tanabe, M., Kawamura, K., Etoh, T. and Ohnuma, N. Neuronal *src* and *trk* A protooncogenes expression in neuroblastomas and patient prognosis. *Int. J. Cancer*, **79**, 226–231 (1998).
16) Brodeur, G. M., Seeger, R. C., Schwab, M., Varmus, H. and Bishop, J. M. Amplification of N-*myc* in untreated human neuroblastomas correlates with advanced disease stage. *Science*, **224**, 1121–1124 (1984).
17) Sugimoto, T., Sawada, T., Matsumura, T., Kemshead, J. T., Ishii, T., Horii, Y., Morioka, H., Morita, M. and Reynolds, C. P. Identical expression of cell surface membrane antigens on two parent and eighteen cloned cell lines derived from two different neuroblastoma metastases of the same patient. *Cancer Res.*, **46**, 4765–4769 (1986).
18) Matsunaga, T., Shirasawa, H., Tanabe, M., Ohnuma, N., Takahashi, H. and Simizu, B. Expression of alternatively spliced *src* messenger RNAs related to neuronal differentiation in human neuroblastomas. *Cancer Res.*, **53**, 3179–3185 (1993).
19) Akiyama, K. and Nishi, Y. Cloning and physical mapping of DNA sequences encompassing a region in N-*myc* amplicons of a human neuroblastoma cell line. *Nucleic Acids Res.*, **19**, 6887–6894 (1991).
20) Stanton, L. W., Schwab, M. and Bishop, J. M. Nucleotide sequence of the human N-*myc* gene. *Proc. Natl. Acad. Sci. USA*, **83**, 1772–1776 (1986).
21) Zaman, G. J. R., Versantvoort, C. H. M., Smit, J. J. M., Eijdems, E. W. H. M., de Haas, M., Smith, A. J., Broxterman, H. J., Mulder, N. H., de Vries, E. G. E., Baas, F. and Borst, P. Analysis of the expression of MRP, the gene for a new putative transmembrane drug transporter, in human multidrug resistant lung cancer cell lines. *Cancer Res.*, **53**, 1747–1750 (1993).
22) Kool, M., de Haas, M., Scheffer, G. L., Scheper, R. J., van

Eijk, M. J. T., Juijn, J. A., Baas, F. and Borst, P. Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Res.*, **57**, 3537–3547 (1997).

23) Zimmerman, K. A., Yancopoulos, G. D., Collum, R. G., Smith, R. K., Kohl, N. E., Denis, K. A., Nau, M. M., Witte, W. N., Toran-Allerand, D., Gee, C. E., Minna, J. D. and Alt, F. W. Differential expression of *myc* family genes during murine development. *Nature*, **319**, 780–783 (1986).
24) Nisen, P. D., Waber, P. G., Rich, M. A., Pierce, S., Garvin, J. R., Jr., Gilbert, F. and Lanzkowsky, P. N-*myc* oncogene RNA expression in neuroblastoma. *J. Natl. Cancer Inst.*, **80**, 1633–1637 (1988).
25) Slavc, I., Ellenbogen, R., Jung, W.-H., Vawter, G. F., Kretschmar, C., Grier, H. and Korf, B. R. *myc* gene amplification and expression in primary human neuroblastoma. *Cancer Res.*, **50**, 1459–1463 (1990).
26) Nakagawara, A., Arima-Nakagawara, M., Scavarda, N. J., Azar, C. G., Cantor, A. B. and Brodeur, G. M. Association between high levels of expression of the *trk* gene and favorable outcome in human neuroblastoma. *N. Engl. J. Med.*, **328**, 847–854 (1993).
27) Tanaka, T., Hiyama, E., Sugimoto, T., Sawada, T., Tanabe, M. and Ida, N. *trk* A gene expression in neuroblastoma. *Cancer*, **76**, 1086–1095 (1995).
28) Matsunaga, T., Shirasawa, H., Tanabe, M., Ohnuma, N., Kawamura, K., Etoh, T., Takahashi, H. and Simizu, B. Expression of neuronal *src* mRNA as a favorable marker and inverse correlation to N-*myc* gene amplification in human neuroblastomas. *Int. J. Cancer*, **58**, 793–798 (1994).
29) Matsunaga, T., Takahashi, H., Ohnuma, N., Tanabe, M., Yoshida, H., Iwai, J., Shirasawa, H. and Simizu, B. Expression of N-*myc* and c-*src* protooncogenes correlating to the undifferentiated phenotype and prognosis of primary neuroblastomas. *Cancer Res.*, **51**, 3148–3152 (1991).
30) Quinn, J. J., Altman, A. J., Robinson, H. T., Cooke, R. W., Hight, D. W. and Foster, J. H. Adriamycin and cisplatin for hepatoblastoma. *Cancer*, **56**, 1926–1929 (1985).