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 tumors are representative childhood cancers for which combined chemotherapy including cisplatin and doxorubicin is routinely performed. The prognostic value 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 MDRI gene.1 Although MDRI expression in relation to the MDR phenotype in certain cancers is well-characterized, evidence concerning the relation of MDRI expression to the clinical behavior of neuroblastomas is contradictory.2,3 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.4,5 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 MDRI 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
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-117 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.19 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.19 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:

- MRP, cMOAT (normal tissues) and N-myc (neuroblastoma cell lines) amplification mixture was subjected to electrophoresis on 2.5% agarose gels.

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 MMRP 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-
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-my c expression were restricted to the N-my c-amplified neuroblastoma cell lines (IMR32, RT-BM-1, NB-1, GOTO, cNBI and LA-N-5), and very low or undetectable N-my c expression was found in the non-N-my c-amplified neuroblastoma cell lines (SK-N-SH, NB69, NB69N and NB69S) and in the normal tissues, as reported previously. 

In the analyses of the 42 primary neuroblastoma specimens, the expression of MRP and N-my c was observed in...

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

| Tissue Type                | MRP        | cMOAT      | N-my c |
|---------------------------|------------|------------|--------|
| kidney (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     |

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**Fig. 1.** Representative results of competitive RNA-PCR analyses for the expression of the target (MRP, cMOAT and N-my c) 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-my c) 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-my c amplification; 3, metastatic disease with N-my c amplification; liver tumor 1, resectable hepatoblastoma; 2, unresectable hepatoblastoma; 3, metastatic hepatocellular carcinoma, adult type.
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 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 ($\chi^2=0.211$, $P=0.6458$ and $\chi^2=2.085$, $P=0.1487$ for MRP and N-myc, respectively, data not shown).

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.

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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 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-feto-protein 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. 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. 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. 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 and others, and these phenomena reduce the prognostic value of N-myc expression for patients with neuroblastomas. 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. 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. The enhanced expression of cMOAT was suggested to be involved in the resistance to cisplatin and other chemotherapeutic drugs in vitro. 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. 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.

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