Advances in Brief

**MDR1 Gene Overexpression and Altered Degree of Methylation at the Promoter Region in Bladder Cancer during Chemotherapeutic Treatment**

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**Abstract**

Overexpression of the multidrug resistance 1 (MDR1) gene is closely associated with the clinical outcome of hematopoietic malignancies, but the alteration of its expression during chemotherapeutic treatment and the precise mechanism underlying MDR1 gene overexpression in solid tumors remains unclear. We determined the expression and degree of methylation at the promoter of the MDR1 gene in bladder cancer. The mRNA levels of the MDR1 gene were found to be markedly enhanced, 3.5- to 5.7-fold higher in bladder cancers after chemotherapeutic treatment than those in untreated primary tumors. The MDR1 gene was overexpressed in recurrent tumors in 89% of patients who showed re-recurrence, whereas overexpression was observed in 25% of the patients without re-recurrence. A statistically significant inverse correlation existed between MDR1 gene overexpression and the methylation of 5′CpG sites at the promoter in patients with bladder cancer after chemotherapeutic treatment, with the degree of methylation at several CpG sites, rather than other specific sites, involved in this regulation. Consistent with the increase in MDR1 expression, the frequency of patients with a hypermethylated promoter decreased to 50 and 17% after intravesical and systemic chemotherapy, respectively. Thus, overexpression of the MDR1 gene might be a prognostic marker for intravesical recurrence, whereas methylation of the promoter region negatively regulates MDR1 expression and the appearance of multidrug resistance mediated by P-glycoprotein in bladder cancers.

**Introduction**

The appearance of tumor cells resistant to multiple anticancer agents is a serious obstacle in cancer treatment. Such a multidrug resistance phenotype is often associated with increased expression of two representative ATP binding cassette superfamily proteins, P-gp, and the MRP (1–4). In particular, studies (5, 6) have described the normal and pathological function of P-gp since the first report by Debenham et al. (5) that demonstrated its participation in producing the multidrug resistance phenotype. P-gp participates in drug resistance against a wide variety of anticancer agents, such as Vinca alkaloids (vincristine, vinblastin), anthracyclines (doxorubicin, daunorubicin), epipodophyllotoxin (etoposide, teniposide), taxols, and actinomycin D (7). The expression of multidrug resistance 1 (MDR1) gene/P-gp is one of most critical molecular targets for limiting drug sensitivity when many human cancer cells from different tumor types are screened (8).

The MDR1 gene/P-gp is expressed not only in various normal human tissues or organs (9) but also in various malignant tumors (10, 11). To develop MDR1/P-gp as a diagnostic marker for additional therapeutic improvement with fewer side effects, one critical question regards how and when the MDR1 gene is specifically expressed in human malignancies during clinical courses. Gene rearrangements at the 5′-flanking region of the MDR1 gene cause gene activation in multidrug-resistant breast and colon cancer cells as well as in human lymphomas, but analysis of the molecular basis for the gene rearrangements in clinical samples remains to be performed (12, 13). The Y-box (inverted CCAAT box) binding protein (YB-1) has an essential role in human MDR1 gene transcriptional activation in cultured cancer cells in the presence or absence of genotoxic stress (14, 15). Intracellular localization of YB-1 in either the cytoplasm or the nucleus appears to be critical for P-gp expression in some human malignant tumors such as breast cancers, osteosarcoma, and ovarian cancers (16–18). On the other hand, the degree of methylation at CpG sites on the MDR1 promoter also plays a key role in MDR1 gene expression. The presence or absence of methylation at CpG sites is closely associated with transcriptional activation of the MDR1 gene in various cultured cell lines (19, 20). Overexpression of the MDR1 gene is inversely corre-

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The abbreviations used are: P-gp, P-glycoprotein; MRP, multidrug resistance protein; RT, reverse transcription; YB-1, Y-box binding protein.
lated with DNA methylation at CpG sites at the 5′-flanking region in chronic lymphocytic leukemias (21) and acute myeloid leukemias (22). These findings suggest that increased expression of the MDR1 gene/P-gp in some human malignancies is induced as a result of changes in either the intracellular localization of YB-1 or methylation of the MDR1 promoter.

Overexpression of P-gp has been found in human bladder cancer cells selected by drug resistance against P-gp-targeting drugs (23–25). In patients with bladder cancers, expression of P-gp is often increased after chemoradiotherapeutic treatment (26). Chemotherapeutic treatments such as systemic chemotherapy (methotrexate, vincristine, doxorubicin, and cisplatin combined treatment) have been effective for advanced bladder cancer, and prophylactic intravesical instillation chemotherapy using anthracyclines (doxorubicin, epirubicin, and terarubicin) has been effective for preventing recurrence of superficial bladder cancer. However, patient responses against advanced bladder cancers are still of relatively short duration, and the recurrence of superficial bladder cancers is still often observed even after intensive intravesical chemotherapy. In this study, we examined whether the degree of methylation at the MDR1 promoter and Absence of chemotherapeutic treatment in bladder cancers. The degree of methylation at the promoter site of the MDR1 gene expression in bladder cancer cells selected by drug resistance against P-gp-targeting drugs (23–25). In patients with bladder cancers, expression of P-gp is often increased after chemoradiotherapeutic treatment (26). Chemotherapeutic treatments such as systemic chemotherapy (methotrexate, vincristine, doxorubicin, and cisplatin combined treatment) have been effective for advanced bladder cancer, and prophylactic intravesical instillation chemotherapy using anthracyclines (doxorubicin, epirubicin, and terarubicin) has been effective for preventing recurrence of superficial bladder cancer. However, patient responses against advanced bladder cancers are still of relatively short duration, and the recurrence of superficial bladder cancers is still often observed even after intensive intravesical chemotherapy. In this study, we examined whether the degree of methylation at the promoter site of the MDR1 gene expression in bladder cancers and also whether chemotherapeutic treatment affects both the degree of methylation at the MDR1 promoter and Absence of chemotherapeutic treatment in bladder cancers.

Materials and Methods

Patients, Samples, and Cell Line. The study used 51 clinical samples from 49 patients with bladder cancer who underwent resection of bladder cancer in Kyushu University Hospital or in Oita Medical University Hospital between September 1991 and June 1998. Table 1 shows the clinicopathologic characteristics of the 51 clinical samples. All of the patients with residual bladder cancer had received systemic chemotherapy, i.e., combined treatment using methotrexate, vincristine, doxorubicin, and cisplatin, or their recurrent tumors had received prophylactic intravesical instillation chemotherapy with either doxorubicin, epirubicin, or terarubicin before surgery. Tumor tissue samples were obtained under an Institutional Review Board-approved protocol, with subjects providing informed consent. Tumor samples were frozen in liquid nitrogen and were stored at −80°C until RNA and DNA extraction. SNK57 cells derived from human bladder cancer were cultivated as previously described (27).

Isolation of DNA and RNA. DNA was isolated from the tissues of patients, using the Easy DNA Kit (Invitrogen Corporation, San Diego, CA) according to the manufacturer’s protocol. RNA was isolated using the RNA extraction reagent, TRIzol (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer’s protocol.

Semi-quantitative RT-PCR Analysis. The PCR in this study was a modification of a previously described method (22). The PCR primers were 5′-CACGTTGTGTGGAAGCTAACC-3′ and 5′GAAGGCAGAGCATAAGATGC-3′ for the human MDR1 gene and 5′-GTGGAGCATTCAGACTTGTCTTTCAGC-3′ and 5′TCTACTC AATCAAATTGGGCACTTTTC-3′ for human β2-microglobulin. To quantify human MDR1 and human β2-microglobulin mRNA using RT-PCR (28, 29), the patients’ cDNA samples were diluted serially in water, from 50 to 3 ng/μl for the human MDR1 gene and from 25 to 0.04 ng/μl for the human β2-microglobulin mRNA, and were mixed to a final volume of 5 μl with 1 μM primer pairs and 1 μl of Taq DNA polymerase. The PCR products were separated by electrophoresis on 3% agarose gels, which were then stained with ethidium bromide and visualized under ultraviolet light.

Table 1 Clinicopathological parameters (n = 51)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of patients</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>12</td>
</tr>
<tr>
<td>Age (y)</td>
<td>Median</td>
<td>67</td>
</tr>
<tr>
<td>T stage</td>
<td>T2</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>21</td>
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<td></td>
<td>T3b</td>
<td>9</td>
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<tr>
<td></td>
<td>T3a</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>T2b</td>
<td>3</td>
</tr>
<tr>
<td>Grade of tumor</td>
<td>G1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>G2</td>
<td>13</td>
</tr>
<tr>
<td>Clinical status</td>
<td>Unrelated primary tumor</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Recurrent tumor after TUR-Bt and intravesical chemotherapy</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Residual tumor after systemic chemotherapy</td>
<td>12</td>
</tr>
</tbody>
</table>

* TUR-Bt, transurethral resection of bladder tumor.
* Intravesical chemotherapy is prophylactic intravesical instillation chemotherapy with doxorubicin or its derivatives.
* Systemic chemotherapy is methotrexate, vincristine, adriamycin, and cisplatin combined chemotherapy.
SYBER Green I (Molecular Probe, Eugene, OR) and were examined by means of FLA2000 (Fuji Film, Tokyo, Japan) and image analysis (FLA2000; Fuji Film). We repeated the assay at least twice to confirm its reproducibility. We accurately detected MDR1 gene expression under the condition of an average error of $\pm 18\%$ in each sample.

**RNase Protection Assay.** The RNase protection assay in this study was a modification of a previously described method (22). Transcripts originating from the MDR1 promoter, referred to as the “downstream promoter,” protect 130- and 134-bp fragments of the RNA probe. A 324-nucleotide sequence of the same probe was protected by MDR1 transcripts originating at an “upstream promoter” (29). Twenty-five µg of RNA was hybridized using $2 \times 10^5$ cpm of antisense RNA probe. We evaluated the MDR1 mRNA levels standardized by the $\beta_2$-microglobulin expression. The $\beta_2$-microglobulin cDNA expression was measured using a 206-bp cDNA fragment from human $\beta_2$-microglobulin cDNA subcloned into the pGEM-T vector, and was linearized using PstI.

**Immunohistochemistry.** The immunohistochemistry used in this study was a modification of a previously described method (17). In brief, bladder tumors were fixed in 10% form-
malin and were embedded in paraffin. Histological sections were stained with H&E. P-gp was detected using two kinds of monoclonal antibody, C219 (Centocor, Malvern, PA) and JSB-1 (Sanbio, Uden, the Netherlands).

Quantitative PCR-based Methylation Analysis. The PCR used in this study was a modification of a previously described method (22). In brief, 3 μg each of control and DNA were digested by 300 U of MspI (Fermentas MBI, Vilnius, Lithuania) or HpaII (Takara Shuzo, Kyoto, Japan) at 37°C for 16 h, 1/15 volume of 0.6 M Tris (pH 7.5) and 1.5 M NaCl were added, and the mixture was then digested by 30 U of PstI (Nippon Gene) at 37°C for 8 h. To analyze the degree of methylation of the MDR1 5′CpG promoter region, restriction-digested DNA was analyzed using PCR in 5 μl reactions containing 1 μM each of sense and antisense primers and 1 U of Taq DNA polymerase. The PCR products were separated by electrophoresis on 3% agarose gels, which were then stained with SYBER Green and were analyzed by scanner and image analysis (FLA2000 image; Fuji Film).

Southern Blot Analysis. The degree of methylation of MspI/HpaII sites was investigated by separating genomic DNA that had been digested by HpaII and PstI on a 2.0% NuSieve 3:1 gel and by transferring the DNA fragments to a nylon filter (Hybond N+; Amersham). A 978-bp PstI-PstI fragment of the promoter region of MDR1 was used as a probe (20). This probe contained five MspI/HpaII sites (see “Results”).

Statistical Analysis. Mann-Whitney tests, Kruskal-Wallis tests, and multiple comparisons were used to compare two or more groups. Piecewise linear regression analysis was performed to examine the relationship between the expression and degree of methylation of the MDR1 gene (30). Computations were carried out using BMDP statistical software on a SPARC Station 20 (Los Angeles, CA).

Results

Evaluation of MDR1 Gene Expression in Patients with Bladder Cancer Using Semiquantitative RT-PCR and RNase Protection Assay. Expression of the MDR1 gene was determined by measuring the PCR products after amplifying serially diluted cDNA (Fig. 1A). Different dilutions of cDNA were tested within the exponential range, depending on the level of MDR1 mRNA (Figs. 1, A and B). The diluted series of cDNA from patient 1 (Pt 1) and SNK57 cells were used as templates for the PCR reaction using MDR1 primer pairs.

Figure 1, A and C, shows the results of the FLA2000 quantitative analysis of the PCR products as a function of the dilution rate of input RNA. The relative mRNA levels of the MDR1 gene were calculated on the basis of the dilution rate at the exponential range and were normalized by dividing by the relative β2-microglobulin mRNA level. The β2-microglobulin mRNA levels were almost the same (Fig. 1D). The MDR1 mRNA levels were 4.2-fold higher in patient 1 (Pt 1) than in SNK57 cells (Fig. 1B).

We also performed an RNase protection assay to evaluate the mRNA level of MDR1, with the results correlating well with
the results by RT-PCR for higher expression samples (Fig. 2). For instance, MDR1 expression levels in patients (Pt) 2, 4, 6, 8, 9, 7, 3–1, 5, and 3–2 were 1.2, 1.2, 1.7, 1.7, 4.7, 6.2, 8.3, 11, and 20, respectively, using RT-PCR analysis and were 1, 2, 2, 3, 5, 6, 8, 22, and 30, respectively, using the RNase protection assay (Fig. 2). The MDR1 transcripts of KB-C1 cells overexpressing MDR1 that were used as a positive control were detected as 130/134- and 324-bp fragments as reported by Mickley et al. (12), whereas only 130/134-bp fragments were detected in all of the patients.

We also evaluated the P-gp expression using immunohistochemical analysis. Figure 3, A and B, shows the immunostaining data of P-gp in one patient (patient 5) who showed an MDR1 expression value of 11 as determined by semi-quantitative RT-PCR. In contrast, cells were not stained with anti-P-gp antibody in one patient (patient 2) who had an expression value of 1.2 (Fig. 3, C and D). These immunohistochemical findings were represented by using anti-P-gp antibody C219 (Fig. 3, A and C) and JSB-1 (Fig. 3, B and D). We also analyzed P-gp expression immunohistochemically in other patients who had undergone total cystectomy, using two P-gp antibodies, C219 and JSB-1.

Alteration of MDR1 Expression during Chemotherapeutic Treatment and Correlation with Clinical Outcome.

We determined the amounts of MDR1 mRNA in all of the clinical samples, and these values were normalized when the mRNA level of MDR1 in SNK57 cells derived from bladder cancer was defined as 1.0. The median values of MDR1 mRNA in untreated primary tumors, recurrent tumors after prophylactic intravesical instillation chemotherapy, and residual tumors after systemic chemotherapy were 1.2 (n = 23), 4.2 (n = 16), and 6.8 (n = 12), respectively (Fig. 4).

Because MDR1 expression varied after chemotherapeutic treatment, we next evaluated the possible correlation between MDR1 gene overexpression and clinical outcome in bladder cancer patients. Thirteen of 16 patients who had recurrent tumors after intravesical chemotherapy were treated using transurethral resection of bladder tumor and were followed up for >2 years. Nine of these 13 patients had re-occurrence of cancer. The median MDR1 gene expression and the number of MDR1 positive-patients in this nine-patient group were 4.8 and 8 (89%), respectively, whereas those of four patients who did not have re-occurrence tumor were 2.0 and 1 (25%), respectively, when we defined the “MDR1-positive” samples as 4.0. (Table 2). The MDR1 gene overexpression after chemotherapeutic treatment thus might be a prognostic factor indicating additional recurrence.

Degree of Methylation of the MDR1 Promoter Region.

To reveal the molecular basis of MDR1 overexpression after chemotherapy, we next examined whether the degree of methylation is associated with MDR1 gene expression in patients with bladder cancer. Two primer pairs, MM2 and MM4, which amplify across the MspI/HpaII site, were used to analyze the degree of methylation of the MDR1 gene promoter region (Fig. 5). By contrast, TPI5 that crosses the MspI/HpaII site, which is never methylated, at the triosephosphate isomerase gene promoter region was used as a negative control (Fig. 5).

Southern blot analysis confirmed the results of PCR analysis for heavily methylated patients. In patients 10, 11, and 12, who had a degree of methylation of 47, 68, and 50%, respectively, as determined by PCR assay, undigested fragments of 492, 546, 602, 656, and 978 bp were observed using Southern blot analysis (Fig. 6, A and B). By contrast, we observed only completely digested fragments of 322 and 401 bp in patients 3–1, 13, and 3–2, who had a degree of methylation of 21, 16, and 5%, respectively (Fig. 6B). The results obtained using the PCR assay correlated well with the results of Southern blot analysis for heavily methylated patients. However, we did not detect a low level of methylation using Southern blot analysis, although we detected a low level of methylation using the PCR assay.

De Novo Methylation and Demethylation at Specific CpG Sites in the MDR1 Promoter Region.

We next examined whether alteration of the degree of methylation is site-specific and also whether the altered degree of methylation of specific sites correlates with MDR1 expression. Figure 7 shows the correlation between the degree of methylation at CpG sites analyzed using MM2 primer pairs (MM2 sites, Fig. 5) and MM4 primer pairs (MM4 sites, Fig. 5). No obvious correlation was shown between the degree of methylation at MM2 sites and at MM4 sites statistically in the whole. We detected hypermethylation at MM2 sites with hypomethylation at MM4 sites in some patients, hypomethylation at MM2 sites with hypermethylation at MM4 sites in other patients, and hyper- or hypomethylation at both sites in other patients (Fig. 7). The degree of methylation at
two HpaII clusters, MM2 and MM4 sites, thus appears to be altered independently. Our results indicate that the degree of methylation at both MM2 and MM4 sites should be included in the evaluation of methylation conditions at the MDR1 promoter (see next section). We also analyzed separately the relationship between the degree of methylation at MM2 sites and at MM4 sites in light of the types of therapy used for each primary tumor, residual tumor, and recurrent tumor. However, we could not find any correlation between them.

We next analyzed the site specificity for methylation alteration more precisely using Southern blot analysis. We did not detect (a) 376-, (b) 728-, and (c) 777-bp bands that indicated hypermethylated \( \langle / \rangle \) sites, or (b) 728- and (d) 455-bp bands that indicated a hypomethylated fl site with hypermethylated \( \langle / \rangle \) and/or fi sites (Fig. 6, A and B). Although we did not distinguish \( \langle / \rangle \) and fi because of the short distance, the HpaII \( \langle / \rangle \) site tended to be hypomethylated, and the fi site tended to be hypermethylated. Furthermore, all of the expected bands of 492, 546, 602, and 656 bp were observed in each patient, depending on the degree of methylation of sites fi and ° (Fig. 6). The degree of methylation of the HpaII sites of \( \langle / \rangle \) and fi thus appears to shift randomly, consistent with the results obtained using PCR assay (Figs. 6 and 7). The order of the sites that were demethylated appeared to proceed from site \( \langle / \rangle \) to fi and then to fi, as MDR1 expression increased in patients undergoing chemotherapeutic treatment (Fig. 6).

**Correlation between MDR1 Gene Expression and Degree of Methylation at the MDR1 Promoter Region.** We next examined if MDR1 expression is correlated with the degree of methylation at the promoter region. Using Southern blot analysis, we determined that MDR1 expression correlated inversely with the degree of methylation except in several clinical samples of untreated primary tumors that showed a low MDR1 expression and hypomethylation. Using RT-PCR analysis, the level of MDR1 expression in patients with untreated primary tumors (\( n = 23 \)) was lower than 5.0, and the level of methylation assessed at both MM2 sites and MM4 sites varied from 2.2 to 115%, which was the sum of the methylation values at MM2 sites and at MM4 sites (Fig. 7). MDR1 expression was low in untreated primary tumors, regardless of the degree of methylation.

We then analyzed the correlation between the degree of methylation and expression of the MDR1 gene in patients after the completion of chemotherapeutic treatment. Figure 8 shows

<table>
<thead>
<tr>
<th>Conditions and clinical outcome</th>
<th>No. of patients with positive ( a ) MDR1 in recurrent tumor ( b )</th>
<th>Median value of MDR1 gene expression in recurrent tumor ( b )</th>
<th>Median follow-up period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Re-recurrence (–) (( n = 4 ))</td>
<td>1 (25%)</td>
<td>2.0</td>
<td>43 months (38–49 months)</td>
</tr>
<tr>
<td>Re-recurrence (+) (( n = 9 ))</td>
<td>8 (89%)</td>
<td>4.8</td>
<td>13 months( ^c ) (6–25 months)</td>
</tr>
</tbody>
</table>

\( a \) Positive, >4.0.

\( b \) MDR1 expression was determined as described in the legend to Fig. 3.

\( c \) The period was from first recurrence to second recurrence.
the correlation between MDR1 gene expression and degree of methylation of 5′CpG sites at the MDR1 promoter region as assessed by MM2 primer pairs together with MM4 primer pairs. Similar correlations were obtained when the degree of methylation was assessed either at MM2 sites alone or MM4 sites alone (data not shown). We also evaluated the degree of methylation at the MM2 and MM4 sites by calculation: \( a \times \text{MM2} + b \times \text{MM4} \), where \( a = 3.1 \) and \( b = 1.2 \); the maximum correlation between the expression of the MDR1 gene and the degree of methylation was \(-0.7515\) and \( p < 0.001 \). With \( a = 1.00 \) and \( b = 1.00 \), the correlation was \(-0.7263\) and \( p < 0.001 \). The results obtained by these two calculations were similar, and we presented the result obtained by introducing \( a = 1.00 \) and \( b = 1.00 \), rather than \( a = 3.1 \) and \( b = 1.2 \).

We then evaluated the graph plot using piecewise linear regression analysis. The regression line was as follows: when \( ST < 17.5\% \), \( MDR1 = -0.91ST + 22.6\); when \( 17.5\% < ST \), \( MDR1 = -0.1ST + 8.4 \), where \( ST \) is the proportion of degree of methylation and MDR1 is the value of MDR1 gene expression. As a result, an inverse correlation was observed quantitatively in patients having undergone chemotherapeutic treatment.

These results strongly indicate an inverse correlation between expression and the degree of methylation of promoter CpG sites of the MDR1 gene in bladder cancer after chemotherapeutic treatment.

Alteration of the Degree of Methylation during the Clinical Course. Knowing if the degree of methylation affects the expression of the MDR1 gene during a clinical course for bladder cancer is important. We defined the cutoff point as 33.5%, which was the median value of the degree of methylation at the promoter region. We evaluated three groups of patients undergoing a clinical course for treatment of bladder cancer. In the first group, composed of patients with untreated primary tumors with intravesical recurrence, 9 (82%) of 11 showed hypermethylation, whereas the median value of the MDR1 gene expression was 0.9. The second patient group had recurrent tumors after intravesical chemotherapy, with a median MDR1 expression value of 4.2 (\( n = 16 \)) and 8 (50%) of 16 patients showing hypermethylation. The number of patients with a hypermethylated MDR1 promoter thus decreased from 82 to 50% during the interval between intravesical chemotherapy and recurrence. The third group used systemic chemotherapy. The median value of MDR1 expression was 6.8 (\( n = 12 \)) and 2 (17%) patients of 12 showed hypermethylation. Again, the MDR1 promoter was demethylated, and MDR1 expression increased after systemic chemotherapy compared with before the treatment.

Samples for each patient before and after chemotherapeutic treatment were available from four patients. In all of the cases, the degree of methylation decreased, and MDR1 expression increased during the clinical course lasting from the first detection of the untreated primary tumor to tumor recurrence or residual tumor (Table 3). In cases 1 and 2 (see Table 3), MDR1 gene expression was very low when the promoter region was hypermethylated in primary tumors. In recurrent tumors after intravesical chemotherapy, MDR1 gene expression increased.
and the promoter region was hypomethylated (Table 3). In case 3, \textit{MDR1} gene expression increased 8.0-fold in residual tumors after systematic chemotherapy, whereas the degree of methylation decreased only slightly to 60% of the initial level. In case 4, the residual tumors after systemic chemotherapy showed a 2.4-fold increase in \textit{MDR1} mRNA levels compared with \textit{MDR1} mRNA levels at initial recurrence after intravesical chemotherapy. Thus, about a threefold decrease in the degree of methylation appeared in residual tumor after systemic chemotherapy in case 4.

**Discussion**

In this study, we used semi-quantitative RT-PCR analysis to evaluate the \textit{MDR1} mRNA/P-gp expression level of 51 clinical samples from patients with bladder cancer. Our clinical data showed that the median value of \textit{MDR1} gene expression was 1.2 in untreated primary tumors, whereas in recurrent tumors after intravesical chemotherapy it was 4.2, and in residual tumors after systemic chemotherapy the value was 6.8. Furthermore, we showed that \textit{MDR1} gene overexpression after chemotherapeutic treatment could be a prognostic factor indicating additional recurrence. Naito \textit{et al.} (31) reported that verapamil, a known chemosensitizing agent of multidrug resistance mediated by P-gp, could enhance the preventative effect of anthracyclines against intravesical recurrence in a randomized study of 157 clinical outcomes. The expression of the \textit{MDR1} gene thus increased after chemotherapy, and this increased expression of \textit{MDR1} might be partly involved in drug resistance and intravesical recurrence in patients with bladder cancer.

We then asked what determines \textit{MDR1} overexpression, and we found an inverse correlation between \textit{MDR1} overexpression and the degree of methylation at CpG sites of the \textit{MDR1} promoter region. Alteration of the degree of methylation at the promoter region of \textit{MDR1} appears to progress in two steps during a clinical course. In the majority of patients with bladder cancer, the \textit{MDR1} gene is \textit{de novo} methylated during carcinogenesis from the hypomethylation level in normal bladder tissue (data not shown) by means of increased DNA methyltransferase activity or by other mechanisms (32). Thus, demethylation occurs during chemotherapeutic treatment, and the \textit{MDR1} gene is overexpressed after chemotherapeutic treatment attributable to cytotoxic stress caused by anticancer drugs and/or the selection of cancer cells with hypomethylation and a high \textit{MDR1} expression. We found that the degree of methylation changed from the hypermethylation level to the hypomethylation level, which was accompanied by overexpression of the \textit{MDR1} gene, in all of the patients whose specimens were available both before and after chemotherapeutic treatment (Table 3).

Regardless of a slight degree of site specificity, such as

<table>
<thead>
<tr>
<th>Condition</th>
<th>Degree of methylation (MM2 % + MM4 %)</th>
<th>\textit{MDR1} gene expression</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>Primary tumor</td>
<td>54</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Recurrent tumor</td>
<td>16</td>
<td>4.2</td>
</tr>
<tr>
<td>Case 2</td>
<td>Primary tumor</td>
<td>54</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>Recurrent tumor</td>
<td>25</td>
<td>4.7</td>
</tr>
<tr>
<td>Case 3</td>
<td>Primary tumor (Patient 3-1)</td>
<td>11</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>Residual tumor</td>
<td>7</td>
<td>10.9</td>
</tr>
<tr>
<td>Case 4</td>
<td>Recurrent tumor (Patient 3-2)</td>
<td>21</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>Residual tumor (Patient 3-2)</td>
<td>6</td>
<td>19.9</td>
</tr>
</tbody>
</table>

* Degree of methylation was analyzed using PCR (Fig. 5) and is represented as a percentage.
* \textit{MDR1} expression was determined as described in legend to Fig. 4.
* THP-ADM, pirarubisin.
sites $\Delta$ and $\boxtimes$ for methylation and/or demethylation (see Fig. 5A), the degree of methylation of any Cpg site appeared to correlate inversely with MDR1 expression. Moreover, we found not only a lesser degree of methylation at any specific site or a lesser fraction of cells with hypermethylation but also a smaller number of sites with hypermethylation and more MDR1 expression. In our previous study (21), the degree of methylation detected by MM2 primer pairs at sites $\Delta$ and $\boxtimes$ and 21 bp adjacent to the YB-1 binding site correlates inversely with the MDR1 expression in acute myeloid leukemias, but such an inverse correlation is not observed when determined by MM4 primer pairs. In this study, we found again that sites $\Delta$ and $\boxtimes$ were highly susceptible to demethylation or were highly protected from methylation during overexpression of the MDR1 gene. Transcription factors protect the promoter from methylation (33). Consistent with this fact, Sp1 elements may prevent the spread of methylation (34, 35). In contrast to the results obtained in acute myeloid leukemias (21), in the present study the altered degree of methylation status of other sites such as $\mathbb{A}$ and $\mathbb{B}$ as detected by MM4 primer pairs also correlates with MDR1 expression in chemotreated bladder cancers. The degree of methylation of putative cis-acting elements at or near sites $\mathbb{A}$ and $\mathbb{B}$ in intron 1 may affect the MDR1 expression. Alternatively, the hypermethylation status expands from sites $\mathbb{A}$ and $\mathbb{B}$ to sites $\Delta$ and $\boxtimes$, resulting in overall hypermethylation at the promoter region (see Fig. 5A), including the YB-1 binding site, and inhibition of MDR1 expression. The expansion of the methylated level at CpG sites with alteration of the chromatin structure may be a mechanism for transcriptional inhibition both in vivo and in vitro systems (19, 36, 37).

In conclusion, MDR1 gene overexpression might be a prognostic factor for intravesical recurrence, and the degree of methylation in the MDR1 promoter region appears to be associated closely with MDR1 gene expression. The hypomethylation status of the MDR1 promoter might be a necessary condition for increasing MDR1 mRNA levels, as well as for developing a multidrug resistant phenotype, in patients with bladder cancer.

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References


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