Regulations of *ABCB1* and *ABCG2* Expression Through MAPK Pathways in Acute Lymphoblastic Leukemia Cell Lines

HIROTAKA TOMIYASU1, MANABU WATANABE2, KANJI SUGITA3, YUKO GOTO-KOSHINO1, YASUHITO FUJINO1, KOICHI OHNO1, SUMIO SUGANO2 and HAJIME TSUJIMOTO1

1Department of Veterinary Internal Medicine, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo, Japan; 2Laboratory of Functional Genomics, Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Minato-ku, Tokyo, Japan; 3Department of Pediatrics, School of Medicine, University of Yamanashi, Chuo, Japan

Abstract. Background: One of the major causes of failure in chemotherapy for patients with acute lymphoblastic leukemia (ALL) is the acquisition of multidrug resistance (MDR). Predominant mechanisms for MDR acquisition include the overexpression of efflux pumps. In the present study, the regulation of the expression of two genes that encode efflux pumps, ATP-binding cassette, sub-family B, member 1 (*ABCB1*) and *ABCG2*, through mitogen-activated protein kinase (MAPK) pathways was examined. Materials and Methods: *ABCB1* and *ABCG2* mRNAs were quantified in a T-ALL cell line, CCRF-HSB-2 and a B-ALL cell line, YAMN90 using real-time RT-PCR. Changes in the mRNA amounts of these genes were examined after activation or inhibition of MAPK/ERK pathway and c-Jun NH2-terminal kinase (JNK) pathway. Results: Activation of MAPK/ERK pathway up-regulated *ABCB1* expression but down-regulated *ABCG2* expression. Activation of JNK pathway up-regulated *ABCG2* gene expression. Conclusion: The expressions of *ABCB1* and *ABCG2* genes were regulated through MAPK/ERK and JNK pathways in the human ALL cell lines.

Acute lymphoblastic leukemia (ALL) is a biologically and clinically heterogeneous group of diseases that are considered to be the result of clonal proliferation of malignant lymphoid progenitors. ALL commonly originates from the B-cell lineage, which accounts for 67.6% of overall incidence of ALL, and T-cell lineage ALL is an uncommon disorder that accounts only for 15.2% of overall incidence of ALL (1). Approximately 25% of children with ALL experience relapse and cannot be cured with current chemotherapy (2). One of the major causes of chemotherapy failure for patients with ALL is intrinsic or acquired multidrug resistance (MDR) (2).

Although various molecules have been reported to contribute to the development of MDR (3, 4), acquisition of MDR is not well-understood. One of the most important mechanisms associated with MDR is the reduction of intracellular concentrations of chemotherapeutic agents due to overexpression of efflux pumps, such as the ATP-binding cassette (ABC) transporters (5). The efflux pumps encoded by *ABCB1*, *ABCC1* and *ABCG2* genes are representative ABC transporters associated with the acquisition of MDR, and overexpression of these genes has been found in various types of tumor cells with MDR phenotypes (6). In hematopoietic tumors, expression of P-glycoprotein (encoded by the *ABCB1* gene) and ABCG2 is frequently observed after chemotherapy in patients with acute myeloid leukemia (AML) (7, 8) and multiple myeloma (9). In ALL patients, high expression of *ABCB1* gene at diagnosis has been reported to be correlated with a poor prognosis (10), and a possible correlation between ABCG2 expression and shorter disease-free survival was also reported (11).

The mechanisms that induce overexpression of these transporters are still unclear. Various intracellular signaling systems have been reported to regulate the expressions of ABC transporters. Mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway (12) and c-JUN NH2-terminal kinase (JNK) pathway (13) were reported to be involved in the regulation of *ABCB1* gene expression. These two MAPK pathways were also reported to regulate the expression of *ABCG2* gene (14, 15). In addition, we recently reported that the
ABCB1 regulation of the expression of four transporter genes, expression has not been studied in ALL cells. However, the association of these pathways with regulation of transporter expression has not been studied in ALL cells.

The purpose of the present study was to examine the regulation of the expression of four transporter genes, ABCB1, ABCC1, ABCG2 and LRP, through MAPK pathways.

Materials and Methods

Cell culture. CCRF-HSB-2 human T-ALL cell line (17), which was provided by RIKEN BRC (Tsukuba, Ibaraki, Japan) through the National Bio-Resource Project of the MEXT, JAPAN, and the human pre-B-ALL cell line YAMN90, which was established at Yamanashi University (18), were used in this study. These cell lines were grown in RPMI-1640 supplemented with 10% fetal calf serum and cultured at 37°C in a humidified atmosphere of 5% CO2.

Relative quantification of efflux pump mRNAs. The expression levels of ABCB1, ABCC1, ABCG2 and LRP genes were relatively quantified by a real-time reverse transcriptase-polymerase chain reaction (RT-PCR) system as reported in our previous study (19) with the primer pairs listed in Table I. To normalize the amount of cDNA sample, the rations of the relative quantities of cDNA for sample, the rations of the relative quantities of cDNA for each gene were adopted. For relative quantification, assay-specific standard curves were prepared, and the relative quantity of each gene was calculated by plotting the quantification cycle in the prepared standard curves. To normalize the amount of cDNA sample, the rations of the relative quantities of cDNA for ABCB1, ABCC1, ABCG2 and LRP genes to that of ABL1 gene were adopted. Real-time RT-PCR assays were performed in triplicate.

Changes in the expression levels of transporter genes due to activation of MAPK/ERK and JNK pathways. For activation or inhibition of MAPK/ERK and JNK pathways, phorbol 12-myristate 13-acetate (PMA) (protein kinase C activator, Wako, Osaka, Japan), U0126 (MAPK/ERK kinase inhibitor; Promega, Leiden, the Netherlands), and SP600125 (JNK inhibitor; Promega), were used as previously described (15, 21, 22). Briefly, CCRF-HSB-2 and YAMN90 cell lines untreated or pre-incubated with 10 μM U0126 for 1 h or 50 μM SP600125 for 40 min were cultured in the presence of 200 nM PMA for 8 h. The relative quantity of ABCB1, ABCC1, ABCG2 and LRP mRNA in these cells was then compared to those in non-treated cells using real-time RT-PCR as described above. The fold-change in the relative quantity of each mRNA in treated cells was calculated by comparing it to that in non-treated cells.

Western blotting for MAPK/ERK and JNK pathway proteins. For confirmation of activation or inhibition of MAPK/ERK and JNK pathways by PMA, U0126 and SP600125, western blotting analysis was performed as described in our previous study (16). In brief, CCRF-HSB-2 and YAMN90 cells untreated or pre-incubated with U0126 or SP600125 were cultured in the presence of 200 nM PMA for 30 min, and whole-cell lysates were extracted. Equal amounts of the extracted proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in 5%-skimmed milk/tris-buffered saline with Tween20, and then incubated with primary antibodies against phospho-ERK1/2 (1:2,000), ERK1/2 (1:1,000), phospho-JNK (1:1,000), JNK (1:1,000), phospho-c-JUN (1:1,000), c-JUN (1:1,000), and β-actin (1:1,000) (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. After incubation with a secondary antibody of horseradish peroxidase-labeled anti-rabbit IgG (1:3,000; Bio-Rad Laboratories, Hercules, CA, USA) at 1 h at room temperature, immunoreactivity was detected using Luminata Forte Western HRP Substrate (Millipore, Billerica, MA, USA) and visualized using ChemiDoc XRS Plus (Bio-Rad Laboratories).

Rhodamine-123 efflux test. To examine the changes in the function of transporters following the activation or inhibition of MAPK/ERK and JNK pathways, rhodamine-123 efflux test was performed as described in our previous study (23). One million cells of each cell line with or without treatment of PMA alone or combination of PMA and U0126, as described above, were incubated with 200 ng/ml rhodamine-123 (Sigma-Aldrich, St. Louis, MO, USA) in RPMI-1640 medium at 37°C for 20 min. After washing twice with phosphate-buffered saline (PBS), the cells were incubated in rhodamine-123-free medium at 37°C for 60 min, either with or without 2 μM cyclosporin. After incubation, the cells were washed with ice-cold PBS three times, placed on ice in the dark, and

Table I. Primer pairs used for real-time reverse transcription-polymerase chain reaction measurement of the relative quantity of each gene.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primer</th>
<th>Reverse primer</th>
<th>Amplicon size (bp)</th>
<th>Genebank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCB1</td>
<td>5'-AGGCCAACAATACGCTGCTTC-3' (3945-3964)</td>
<td>5'-CCACAGAGAGCTGATTCC-3' (4010-4029)</td>
<td>85</td>
<td>NM_000927</td>
</tr>
<tr>
<td>ABCCI</td>
<td>5'-CCTGTTAACGTCACTTGTTG-3' (3466-3485)</td>
<td>5'-AGGCCAAGTAACACTCTGGA-3' (3561-3580)</td>
<td>115</td>
<td>NM_0009696</td>
</tr>
<tr>
<td>ABCG2</td>
<td>5'-TTGCGCTTGCAACAACTTGTTG-3' (785-804)</td>
<td>5'-TCACAGCACACCCAGGATAAA-3' (894-912)</td>
<td>128</td>
<td>NM_01257386</td>
</tr>
<tr>
<td>LRP</td>
<td>5'-TGAGGAGGTTCTGGATTTGG-3' (779-798)</td>
<td>5'-TGCACTGTTACCCACACTG-3' (894-913)</td>
<td>135</td>
<td>NM_005115</td>
</tr>
<tr>
<td>ABL1</td>
<td>5'-TGAGGAGGTTCTGGATTTGG-3' (779-798)</td>
<td>5'-TGCACTGTTACCCACACTG-3' (894-913)</td>
<td>135</td>
<td>NM_005157</td>
</tr>
</tbody>
</table>

ABL1: c-abl oncogene 1. The numbers in the parenthesis indicate the nucleotide numbers registered in GenBank.
subjected to flow cytometric analysis. Cells that had not been exposed to rhodamine-123 were used as negative controls. A canine lymphoma cell line, Ema, which exhibits MDR and expresses ABCB1 (23), was used as a positive control. Twenty thousand events were counted using a FACSCalibur™ instrument (Becton Dickinson, San Jose, CA, USA) and analyzed with FlowJo software (Tomy Digital Biology, Tokyo, Japan). Rhodamine-123 efflux index (REI) was calculated as \[ \frac{\text{the mean fluorescence intensity (MFI) of the sample incubated with rhodamine-123 and cyclosporin} - \text{the MFI of the negative control}}{\text{the MFI of the sample incubated with rhodamine-123} - \text{the MFI of the negative control}} \]. The rhodamine-123 efflux test was performed in triplicate.

**Statistical analysis.** The non-parametric Mann-Whitney U-test was used for comparison of relative quantities of genes. All statistical tests were two-sided and were performed using JMP version 5.0.1 (SAS Institute, Cary, NC, USA). For all tests, \(p<0.05\) was used as the level of significance.
Results

Relative quantification of efflux pump mRNAs. In real-time RT-PCR, the relative expression levels of \(ABCB1, ABCC1, ABCG2\) and \(LRP\) genes were measured in CCRF-HSB-2 and YAMN90 cells (Figure 1). Among these four genes, the expression of \(ABCC1, ABCG2\) and \(LRP\) mRNAs was detected in both cell lines, whereas the expression of \(ABCB1\) mRNA was not observed in either of the cell lines.

MAPK/ERK pathway regulated \(ABCB1\) and \(ABCG2\) gene expression. In both CCRF-HSB-2 and YAMN90 cells, the quantity of phospho-ERK1/2 markedly increased by treatment with PMA, and this increase was partially inhibited by treatment with U0126 (Figure 2). After treatment with PMA, the amount of \(ABCB1\) mRNA increased; it decreased after treatment with U0126 in both cell lines (Figure 3A). The expression of \(ABCG2\) gene significantly increased by treatment with PMA in CCRF-HSB-2 cells (2.3-fold; Figure 3B) compared with that in untreated cells. In addition, the quantity of \(ABCG2\) mRNA further increased (28-fold) by combined treatment with PMA in CCRF-HSB-2 cells (2.3-fold; Figure 3B) compared with that in untreated cells. In addition, the quantity of \(ABCG2\) mRNA was not observed after treatment with PMA or the combination of PMA and SP600125 in YAMN90 cells.

\(ABCC1\) and \(LRP\) genes did not change in either of the two cell lines when treated with PMA or the combination of PMA and U0126.
**Discussion**

The present study showed that in human ALL cell lines, expression of ABCB1 gene was regulated through MAPK/ERK pathway and that of ABCG2 gene was regulated through MAPK/ERK and JNK pathways.

In both CCRF-HSB-2 and YAMN90 cells, mRNA expression of ABCB1 gene was detected after treatment with PMA, a protein kinase C activator, although the expression of ABCB1 gene was not observed before treatment. In addition, this increase in ABCB1 gene expression was inhibited by treatment with U0126, a MAPK/ERK kinase inhibitor. These findings indicated that the expression of ABCB1 gene was up-regulated through the MAPK/ERK pathway in these two ALL cell lines. This observation agreed with findings of previous studies using human B-cell lymphoma cells or canine lymphoma cells (12, 16).

The expression of ABCG2 gene increased after treatment with both PMA, and U0126, and expression was further increased by treatment with the combination of PMA and U0126 in CCRF-HSB-2 cells. In contrast, the expression of this gene apparently decreased after treatment with PMA, and this decrease was inhibited by treatment with U0126 in YAMN90 cells. These results indicate that ABCG2 gene expression was down-regulated through the MAPK/ERK pathway, and that some other pathways up-regulated the expression of this gene. Based on this possibility, the role of the JNK pathway in the regulation of the expression of ABCG2 gene was examined. In CCRF-HSB-2 cells, the JNK pathway was activated by treatment with PMA and was inhibited by treatment with the combination of PMA with SP600125. The large increase in ABCG2 gene expression that was observed when CCRF-HSB-2 cells were treated with PMA and U0126 was abolished when cells were treated with PMA, U0126 and SP600125. However, in YAMN90 cells, activation of the JNK pathway was not observed, despite treatment with PMA, and the expression of ABCG2 gene was not significantly different between the cells treated with PMA and U0126 and those treated with PMA, U0126 and SP600125. These results indicate that the JNK pathway up-regulated the expression of ABCG2 gene in CCRF-HSB-2 cells. In previous studies, it was shown that the transcription of ABCG2 gene was down-regulated through MAPK/ERK pathway in a human breast cancer cell line (14) and the expression of this gene was up-regulated through JNK pathway in human colon cancer (15). The findings in the present study were consistent with the results of these previous studies. However, there has been no study revealing the simultaneous inverse regulation of ABCG2 gene expression through MAPK/ERK and JNK pathways as shown in the present study.

The expression of ABCC1 and LRP genes did not change after treatment with PMA and U0126 in the present study. The MAPK/ERK pathway was previously shown to be essential for collagen-induced ABCC1 gene expression in human T-ALL cell lines (24). Combined with the results in the present study, it is reasonable to consider that the MAPK/ERK pathway might be associated with the regulation of ABCC1 gene expression in cooperation with other mechanisms in human T-ALL cell lines, although activation of the MAPK/ERK pathway alone did not increase the ABCC1 gene transcription. Our previous study showed that the MAPK/ERK pathway up-regulated LRP gene expression in canine lymphoma cell lines (16). The reason for the differences between the results of our present study and those obtained in a previous study (16) is not clear;
however, it is possible that the state of activation of factors downstream of ERK differed between human ALL cell lines and canine lymphoma cell lines after treatment with PMA.

In the present study, the relative quantity of mRNA of ABCB1 and ABCG2 genes in CCRF-HSB-2 cells increased after treatment with PMA or the combination of PMA and U0126, and ABCB1 gene expression increased after treatment with PMA in YAMN90 cells. However, in the rhodamine-123 efflux test, it was shown that the function of the efflux pumps was not enhanced despite the increase of the amount of ABC transporter gene mRNAs after treatment with PMA or the combination of PMA and U0126. Previous studies showed that inhibition of the MAPK/ERK pathway, which up-regulated ABCB1 gene, or inhibition of the JNK pathway, which up-regulated ABCG2 gene, could reduce the function of transporters encoded by these genes (12, 15), indicating that the inhibition of these pathways could re-establish sensitivity to chemotherapeutic agents in tumor cells with an MDR phenotype. However, the results of the present study raise the possibility that the activation of MAPK/ERK or JNK pathways alone cannot up-regulate the function of ABCB1 and ABCG2 sufficiently to confer MDR phenotype on the ALL cells.

In conclusion, the present study revealed the regulation of ABCB1 and ABCG2 genes through MAPK/ERK and JNK pathways in human ALL cell lines. However, it is suggested that other mechanisms might be important for enhancement of transporter function during MDR acquisition in tumor cells lacking ability for drug efflux. Further study is needed to elucidate the mechanisms of MDR acquisition via overexpression of efflux pumps in addition to the mechanism revealed in the present study.

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References


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