Expression of lung resistance protein and multidrug resistance-related protein (MRP1) in pediatric acute lymphoblastic leukemia

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Multidrug resistance (MDR) is a phenomenon by which cells become resistant to unrelated chemotherapeutic agents. The prognostic value that lung resistance protein (LRP) and multidrug resistance-related protein 1 (MRP1) have in the setting of pediatric acute lymphoblastic leukemia (ALL) is controversial. The aim of this study was to investigate the expression of LRP and MRP1 and effect on clinical outcome and prognosis. The mRNA expression of LRP and MRP1 were analyzed in leukemic blasts of 34 pediatric ALL patients. LRP and MRP1 mRNA expression were detected in 41.2% and 35.3%, respectively. Eleven (91.7%) of 12 patients without LRP achieved CR compared with 9 (50.0%) of 18 with LRP expression. Similarly, 11 (100%) of 11 patients without MRP1 expression achieved CR compared with 9 (47.4%) of 19 with MRP1 expression and higher LRP expression rate or MRP1 expression rate was present in patients with relapse than MDR genes negative patients. The expression of either of two genes was associated with poorer 2-year survival. Also, patients expressing both genes had poorer outcomes and had worse 2-year survival. We suggest that MDR expression affects complete remission and survival rates in ALL patients. Thus, diagnosis appears to provide prognostic information for pediatric ALL.

Key words: drug resistance; genetic determination; ALL

INTRODUCTION

Leukemia represents a clonal expansion and arrest at a specific stage of normal myeloid and lymphoid hematopoiesis. Leukemias are the most common childhood cancers, accounting for about 33% of pediatric malignancies. Over the past two decades, major advances have been achieved in the treatment of cancers and this was attributed to highly potent antineoplastic drugs. However, chemo resistance...
(intrinsic or acquired) still hinders the achievement of complete remission and the cure of patients and is a major obstacle in the successful treatment.\(^2\) Such resistance may be present before treatment begins or may develop during chemotherapy. Drug resistance that extends to structurally and functionally unrelated drugs is termed multidrug resistance (MDR).\(^3\)

Several molecular biological mechanisms have been identified as being associated with MDR.\(^4\) Classical MDR is associated with transmembrane protein-mediated efflux of cytotoxic compounds leading to a decreased cellular drug accumulation and toxicity. Several MDR-related drug efflux pumps have been characterized. Most of them belong to the superfAMILY of ATP-binding cassette (ABC) transporters.\(^2\) The most extensively studied MDR protein is the permeability-glycoprotein (P-gp), also named P-170,\(^5\) encoded by the MDR1 gene and is an ATP-dependent pump conferring cross-resistance to a variety of mechanically and structurally unrelated cytotoxic drugs, such as anthracyclines, vinca alkaloids, and epipodophyllotoxins.\(^6\)

During the past 10 years, several new drug resistance-related proteins have been identified.\(^2\) One of these is the multidrug-resistance-associated protein 1 (MRP1), an ATP-dependent efflux pump, which extrudes glutathione-conjugated compounds out of the cell.\(^7\) Currently, the MRP family consists of nine MRP homologues (MRP1–MRP9). The best characterized of them is MRP1\(^8\), which is the only MRP family member associated with clinical MDR.\(^9\) MRP1 is also a member of the ABC transporter protein family.\(^7\) It is a 190 kDa protein encoded by the MDR1 gene, mapped on chromosome 16 (16p13.1).\(^10\) The spectrum of resistance caused by MRP1 overexpression is very similar to that of P-gp\(^11\) with the exception of mitoxantrone.\(^11,12\) These findings suggest that MRP1 plays an important role in the elimination and sequestration of cytotoxic drugs, leading to decreased concentrations at their target sites.\(^2\)

Another mechanism of drug resistance is the intracellular redistribution of drugs without changing their intracellular accumulation.\(^13,14\) Lung resistance protein (LRP) is another MDR-related protein and is identified as the major vault protein (MVP).\(^2\)Vaults are cytoplasmic organelles,\(^15\) a small portion of which is localized in the nuclear membrane and nuclear pore complex.\(^16\) Vaults are thought to be involved in both the vesicular and nucleocytoplasmic transport of drugs.\(^14\) The LRP was initially identified in an anthracycline-resistant, nonsmall cell lung cancer cell line that lacked P-gp overexpression.\(^17\) Unlike P-gp and MRP, LRP is not a member of the ABC transporter family as there are no transmembrane fragments or ATP-binding sites, characteristic for ABC transporters, were identified in its amino acid sequence.\(^2\) It is thought that LRP decreases the effectiveness of cytotoxic drugs, either by regulating nucleocytoplasmic transport of cytotoxic drugs away from the nucleus and/or by involvement in sequestration of cytotoxic drugs in exocytotic vesicles.\(^18\) The LRP gene is located on chromosome 16 (16p11.2), close to the MRP1 gene, and encodes a 110 kDa protein.\(^19\) LRP has been reported to be involved in resistance to vincristine, doxorubicin, and etoposide.\(^20\)

The exact clinical value of the above MDR proteins in childhood ALL is not clear and existing data are conflicting.\(^21\) So, the aim of this work is to investigate the clinical outcome and prognostic value of MRP1 and LRP mRNA expression in children with ALL.

**PATIENTS AND METHODS**

**Patients**

Thirty-four children (14 males and 20 females), initially diagnosed with ALL, were selected from Hematology/Oncology Unit, Pediatric Department, Tanta University Hospital and El-Mansoura University Hospital. The median age was 10.2 years (range 6.5–16 years). Diagnosis and classification of ALL were made according to the French–American–British (FAB) criteria and immunophenotype analyses. The immunophenotyping was pre-B (CD19+, CD22+, CD10−), common ALL (CD19+, CD22+, CD10+), and T-ALL (CD3+, CD5+, CD7+). The clinical data from all patients were obtained, including age at diagnosis, gender, WBC count at diagnosis, blast cells in BM, and lactate dehydrogenase (LDH). The induction chemotherapy regimens were: Vincristine 1.5 mg/kg/m3/week IV (days 0, 7, 14, 21, 28, 35), Doxorubicin 25 mg/m3/week IV infusion (days 0, 7, 14, 21, 28, 35), L-Asparaginase 6000 u/m3 SC on alternate days for 10 doses, and Prednisone 40 mg/m3/day for 6 weeks orally. On day 21, bone marrow aspiration was done. In nonresponding cases, we add Etoposide 100 mg/m3/dose IV (days 22, 25, 39), Cyclophosphamide 750 mg/m3/dose IV infusion (days 22, 25, 29), Ara-cin 100/m3/dose IV (days 22, 25, 29), and high dose methotrexate 6g/m3 over 4 h on day 8.

From all patients, bone marrow and/or peripheral blood samples were collected on EDTA containing vacutainer tubes. Within 24 h after sampling, mononuclear cells were isolated on Ficoll-Isopaque (Nycomed, Oslo, Norway) density gradient by
centrifugation. The cells were preserved in RPMI 1640 supplemented with 10% FCS and 10% DMSO (Merck, Amsterdam, the Netherlands) and stored at −70°C until use.22 Follow-up for all patients was done for 2 years.

**Definition of disease phase and response**
Complete remission (CR) is defined as a cellularity of more than 20% with fewer than 5% blasts in the bone marrow (BM) after induction chemotherapy,5 and relapse is defined by the appearance of one of the following: (1) more than 50% lymphoblasts in a single BM aspirate; (2) progressive repopulation of lymphoblasts in excess of 5% culminating in more than 25% on two or more BM samples separated by 1 week or more; (3) more than 25% lymphoblasts in the BM and 2% or more circulating lymphoblasts; (4) leukemic cell infiltration in extramedullary organs, for example central nervous system or gonads; (5) lymphoblasts in CSF with cell count greater than 5 WBCs/mm³.23,24

**RNA extraction and cDNA synthesis**
Total cellular RNA was isolated from ALL blasts using Rneasy Mini Kit including DNase digestion (Qiagen, Hilden, Germany). The concentration and purity of RNA were measured by UV spectrophotometer at 260 and 280 nm. The integrity and size distribution of total RNA were checked on agarose gel electrophoresis 1.5%. Subsequently, 1 µg RNA was reverse transcribed to single stranded cDNA in 20 µL reverse transcriptase buffer containing 10 mmol/L DTT, 0.5 mmol/L each of dATP, dGTP, dCTP, and dTTP, 200 units of Moloney murine leukemia virus reverse transcriptase, 5 units of RNase inhibitor, and 5 µmol of random hexamers primers (MBI Fermentas, St. Leon-Rot, Germany) and applied to 7000 sequence detection system at 37°C for 30 min, 42°C for 15 min and 94°C for 5 min.22

**Quantitative real-time PCR (TaqMan technology)**
The mRNA expression levels of LRP, MRPI, and the endogenous housekeeping gene GAPDH as a reference were quantified using real-time PCR analysis (TaqMan) on an ABI prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). The sequences of primers LRP, MRPI, and GAPDH are illustrated in Table 1.2,5 Specific PCR products were amplified and detected using dual-fluorescent non-extendable probes labeled with 6-carboxy-fluorescein (FAM), reporter and 6-carboxytetramethylrhodamine (TAMRA), quencher at 5′-end and 3′-end, respectively. We used 4 µL of a 22.5-fold diluted cDNA in each PCR reaction in a final volume of 20 µL containing 900 nmol/L of sense and anti-sense primers, 200 nmol/L of the TaqMan probe, 5 mmol/L MgCl₂, KCl, and Tris-HCl, 0.2 mmol/L dATP, dCTP, dGTP, dTTP, and dUTP, and 0.5 units of AmpliTaq DNA polymerase (qPCR Core Kit, Eurogentech, Seraing, Belgium). The PCR program was 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C.22

For each patient, the relative mRNA expression levels of LRP and MRPI were calculated using the comparative cycle time (Ct) method.20 Briefly, the target PCR Ct values, that is, the cycle number at which emitted fluorescence exceeds 10× the standard deviation of base-line emissions, are normalized to the GAPDH PCR Ct value by subtracting the GAPDH Ct value from the target PCR Ct value. The relative mRNA expression level to the GAPDH for each target PCR was calculated using the following equation:9

\[
\text{Relative mRNA expression} = 2^{-\frac{\text{Ct}_{\text{target}}}{\text{Ct}_{\text{GAPDH}}}}
\]

**Statistical analysis**
Data were analyzed using chi-square or Fisher exact tests. To analyze the expression of the mRNA levels of resistance genes between different groups we used the Mann–Whitney U-test. Survival curves were plotted using the Kaplan–Meier method, and differences were analyzed using log-rank tests. A p-value <0.05 was considered significant. All statistical analyses were performed using SPSS version 12.

**RESULTS**

**Expression of LRP and MRPI mRNA in ALL patients**
In this study, the expression of specific mRNA of LRP and MRPI was investigated in 34 cases of ALL

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**Table 1. Primer sequences for MRPI, LRP, and GAPDH**

<table>
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<tr>
<th>Target</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>MRPI</td>
<td>5′-TACACCGTGCTGCTTTGTCACT-3′</td>
<td>5′-GTCTTGGTACATCGCCATCATACA-3′</td>
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<td>LRP</td>
<td>5′-GTCTTGGGCGCTGAGCTGGTGC-3′</td>
<td>5′-CTTGCGGTCTCTTGGGTCGCTTT-3′</td>
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<tr>
<td>GAPDH</td>
<td>5′-GGTGGTCCTCCTGACTTCAACA-3′</td>
<td>5′-GTTGGTCGAGGGCAAAG-3′</td>
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patients by real-time PCR. The results are shown in Figures 1 and 2. LRP positivity was found in 20 (58.8%) of 34 patients, while MRP1 positivity was found in 22 (64.7%) of 34 patients. The median LRP expression levels were 0.85 (range 0.79–1.06). For MRP1, the median expression levels were 0.71 (range 0.50–1.21) (Table 2).

**Patient characteristics**

Table 3 illustrates the patient characteristics regarding age, sex, WBC counts, LDH level, and blast cells in BM. Immunophenotype analysis is illustrated in Table 4. No differences in the expression levels of LRP or MRP1 were observed with age, sex, WBC counts, LDH activity, or blast cell counts. However,
both LRP and MRP1 expressions were significantly higher in B lineage (including pre-B and common ALL) ALL compared with T-lineage ALL ($p = 0.035$, $p = 0.013$; respectively, Table 4).

**Association of LRP and MRP1 mRNA expression at diagnosis with rate of complete remission, relapse, and survival time**

LRP mRNA expression at initial diagnosis was associated with a lower CR rate after induction chemotherapy, with only 50.0% (9/18) of LRP-positive ALL patients achieving CR, compared to 91.7% (11/12) of LRP-negative patients achieving CR with statistical significance ($p = 0.021$). On the other hand, MRP1 mRNA-positive patients showed the tendency of lower CR rates (9 of 19 patients, 47.37%) than MRP1-negative patients (11 of 11 patients, 100%) with statistical significance ($p = 0.001$) (Table 5). As regarding the relapse rate, the LRP expression rate was higher in patients who relapsed (4/18, 22.2%) than in LRP-negative patients (1/12, 8.3%), but the difference did not reach the statistical significance ($p = 0.318$), which could be due to the small number of patients analyzed. The same pattern was observed for MRP1; higher expression rate was observed for MRP1 in relapsed patients (5/19, 26.3%) compared to MRP1-negative patients (0/11, 00.0%) with statistical significance ($p = 0.023$) (Table 5). Thus, LRP and MRP1 gene expressions could predict which patients would relapse. Kaplan–Meier analysis showed that the median survival for LRP-positive patients was 440 days, while LRP-negative patients did not reach the median survival time. There was a significant difference in the 2-year survival between those two groups ($p < 0.05$) (Figure 3). Similarly, the median survival was 508 days for patients positive for MRP1 expression, while MRP1-negative patients did not reach the median survival time. The difference was

<table>
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<th>Table 2. LRP and MRP1 mRNA expression levels in ALL patients</th>
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<td>LRP (n = 34)</td>
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<td>Negative</td>
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<td>Positive</td>
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<td>Median mRNA expression</td>
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<td>Range</td>
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<th>Table 3. Clinical features of ALL cases with and without LRP or MRP1 mRNA expression</th>
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<td>WBCs (×10^9/L)</td>
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<td>Blast cell count (%)</td>
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<td>LDH (units/L)</td>
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<td>Mann–Whitney’s test</td>
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statistically significant in the 2-year survival between the two groups ($p < 0.05$) (Figure 4). With all of the 34 ALL patients, only 4 patients died during the 2-year course of follow-up (at 136, 204, 247, and 391 days).

Outcome in ALL patients expressing the two MDR genes

Double positivity for LRP and MRP1 mRNA was detected in eight samples (23.5%). While 37.5% (3/8) of ALL patients expressing both LRP and MRP1 mRNA achieved CR, 62.5% (5/8) of those expressing both genes are relapsed. Expressing both LRP and MRP1 mRNA had significant influence on the CR and relapse rates after induction chemotherapy. The median survival of LRP and MRP1-double positive patients was 405 days.

DISCUSSION

MDR is attributed to the overexpression of certain efflux proteins that are capable of trafficking chemotherapeutic drugs out of the cell, like P-gp and MRP1, or away from the nucleus by LRP. The exact clinical value of these MDR proteins in childhood ALL, however, is not clear and existing data are conflicting.  

The present study indicates that MDR gene expression can affect outcomes in ALL patients. LRP gene expression at diagnosis appeared to be associated with resistance to induction chemotherapy. While 91.7% of LRP-negative subjects achieved CR, 50% of the LRP-positive subjects achieved CR ($p = 0.021$) (Table 5), and 2-year survival in LRP-positive patients was less than that in LRP-negative patients ($p < 0.05$, Figure 3). Our results are in accordance with those of Huh et al.  and Saubrey et al.  They suggested that LRP mRNA expression was particularly influential in outcomes following induction chemotherapy in ALL patients and might be a mechanism involved in childhood ALL drug resistance. Also, Fichtner et al. found that LRP-positive patients had a lower probability of remaining in continuous first remission. This correlation between LRP expression and treatment outcome is consistent with suggestions that chemosensitive leukemic cells have low LRP mRNA levels.

In this study, we found LRP mRNA expression rate was higher in relapsed patients (22.2%) than LRP-negative relapsed patients (8.3%, $p = 0.318$)
In the study of Volm et al.\textsuperscript{27} LRP protein expression was found in 47\% of patients with initial ALL and 68\% with relapsed ALL. Patients with LRP expression also showed unfavorable prognosis in that study. Increased LRP protein expression in relapsed childhood ALL patients and an inverse correlation between LRP expression and intracellular daunorubicin concentration was reported from another investigation.\textsuperscript{28} These findings suggest that LRP might contribute to drug resistance in children with ALL.\textsuperscript{2} In contrast, in the study of den Boer et al.\textsuperscript{29} no differences in LRP expression have been reported between initial and relapse ALL samples, only in multiple relapse samples LRP expression was

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure3}
\caption{Relationship between LRP mRNA expression and 2-year survival in acute lymphoblastic leukemia patients.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4}
\caption{Relationship between MRP1 mRNA expression and 2-year survival in acute lymphoblastic leukemia patients.}
\end{figure}
found to be significantly increased. Even so, it has been suggested that children with initial ALL and no detectable LRP expression experience significantly longer relapse-free intervals compared to patients with LRP expression.27

Also, another study in lymphoproliferative disorders has shown a correlation between shorter survival and LRP mRNA expression.30 Similar results were reported in other hematological malignancies. LRP is expressed in 36% of the patients with de novo acute myeloid leukemia (AML), and the complete remission rate is lower and overall survival is significantly shorter in AML patients with LRP expression than in patients without LRP expression in de novo and secondary AML.31 In multiple myeloma,32 LRP expression is found in 61% of the patients and is a significant prognostic factor.

The mechanisms of drug resistance mediated by LRP have been unclear until now. Ohno et al.30 demonstrated that the efflux of doxorubicin (DOX) from nuclei isolated from leukemic cells that overexpressed LRP was enhanced and that this efflux was inhibited by anti-LRP antibody. Moreover, the accumulation of DOX in nuclei isolated from leukemic cells that expressed LRP was enhanced by incubating with anti-LRP antibody. These results indicate that functionally active LRP is expressed in leukemic cells and that the expressed LRP transports DOX from nuclei to cytoplasm. These results are also consistent with the report of Kitazono et al.33 DOX is mainly distributed in the cytoplasm of the cells that express LRP but in the nuclei of cells that have no detectable LRP. LRP was shown to be involved in resistance to DOX and to play an important role in the transport of DOX from the nuclei to the cytoplasm.35 These results suggest that LRP-mediated drug resistance is caused by the transport of anti-cancer drugs from nuclei to cytoplasm.30

As regard MRPI, the present study may indicate that positive MRPI had an impact on clinical outcome in childhood ALL as there is an appreciable trend toward bad prognosis associated with MRPI positive cases. MRPI was expressed in 64.7% of patients at initial diagnosis with a median level of 0.71 (0.50–1.2) (Table 2), and 2-year survival in those patients was less than that in MRPI-negative patients (p < 0.05, Figure 4). Furthermore, we found that MRPI was expressed in 26.3% of relapsed patients compared to MRPI-negative patients (0%) (p = 0.023) (Table 5). Our results are in agreement with those of Huh et al.5 who found that high levels of MDRP1 mRNA expression were significantly associated with poor 2-year survival in ALL patients and considered that patients with high MDRP1 may have poorer outcomes.

Plasschaert et al.22 suggested that high expression of MRPI is associated with unfavorable outcome. Relapsed patients showed a higher expression of MRPI gene than patients who remained in complete remission. Another study on a group of Indian ALL patients reported significantly higher MRPI mRNA expression at relapse than at presentation or remission.34 However, this finding has not been confirmed by others, and MRPI measured at the protein level appeared to have no prognostic importance.29 The gene expression patterns were investigated in relation to in vitro cellular drug resistance to prednisone, vincristine, asparaginase, and daunorubicin and no differences were found in the expression of MRPI gene.35 The discrepancy in the results may be related to the fact that the selection of genes was based on the drug sensitivity assay, which is another approach than clinical end points, such as relapse-free survival as used in study of Plasschaert et al.22 or CR rate and 2-year survival as used in the present study.

In the present study, LRP and MRPI-double positive patients showed reduced remission rates after induction chemotherapy and poorer 2-year survival compared to patients not expressing both of the genes. Our results are in agreement with those of Huh et al.5 Another two studies have previously attempted to determine the impact of simultaneous expression of the two MDR genes, and the conclusions drawn by these studies were not consistent with each other.36,37 Both genes are localized at chromosome 16.38 In different non-P-170 MDR cell lines, a concomitant up regulation of LRP and MRPI has been reported.19 Different studies conducted in leukemia showed also simultaneous overexpression of LRP and P-170 or correlation of MRPI and P-170.36,39 So far, the mechanism of simultaneous expression of ABC transporters and vault proteins like LRP are not clear.8

Furthermore, both LRP and MRPI expression were not correlated to other clinical risk factors of ALL, including WBC, LDH or age, in this study (Table 3). By contrast, we found both expression levels were higher in B-lineage ALL compared to T-ALL (p = 0.035, p = 0.013; respectively) (Table 4). Our results are in accordance with those of Kakihara et al.,40 Saubrey et al.,8 and Ogretmen et al.41 as regard to LRP expression, and with those of Tafuri et al.,42 Steinback et al.,43 Plasschaert et al.,22 and Vander Kolk et al.44 as regard to MRPI expression.
From the present study, we can conclude that outcome in ALL patients can vary depending upon expression of LRP and MRP1 mRNA. Expression of LRP or MRP1 mRNA correlated with a low CR rate and poor survival and also could identify patients with an increased risk for relapse. These results suggest that analysis of MDR gene expression at diagnosis of ALL may provide useful prognostic information. Such data are also likely to assist in determining the mechanisms underlying drug resistance. Additionally, with the discovery of other MDR proteins and the role that these proteins may contribute to clinical resistance should be investigated more intensely as the ultimate reasons for the failure of treatment in pediatric ALL.

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