ORIGINAL ARTICLE ◄

# Multidrug Resistance- related Protein (MRP) and Lung Resistance Protein (LRP) m RNA Expression in Egyptian Patients with Acute Leukemia

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## Abstract

**Background and objectives:** Clinical resistance to chemotherapy is a major obstacle in treatment and an important cause of death in Acute Leukemia.Such resistance is usually associated with the expression of multidrug resistance(MDR) genes. The significance of MDR genes expression is still controversial

**Aim of the work:** We investigated whether multidrug resistance-related protein (MRP) and Lung resistance protein (LRP) m RNA expression are associated with outcomes ,clinical and laboratory findings in acute leukemia patients.

**Patients&methods:** At diagnosis we examined MRP and LRP m RNA expression in peripheral blood samples from 50 Egyptian Acute Leukemia patients (25 myeloid& 25 lymphoblastic) using nested RT-PCR. Ten age matched normal individuals were included as control group.

**Results:** m RNA of MRP and LRP genes were detected in 28/50 (56%) & 22/50 (44%) respectively, while there was double expression of both genes in 18/50 (36%) of acute leukemia patients. There was no statistically significant difference between MRP&LRP positive and negative patients as regards their clinical and laboratory data including age, sex , hemoglobin, platelets, hepatosplenomegally, lymphandenopathy and CNS involvement. As regards Total leucocytic count (TLC) the comparison between positive and negative cases showed high statistically significant difference ($p<0.005$). MRP&LRP m RNA expression at initial diagnosis was associated with a lower CR rate after induction chemotherapy, with only 6/28 (21.4%) of MRP positive acute leukemia patients achieving CR, compared to 15/22 (68.1%) of MRP negative acute leukemia patients achieving CR **(P<0.05),** while 3/22 (13.6%) of LRP positive acute leukemia patients achieved CR compared to 18/28 (64.3%) of LRP negative acute leukemia patients achieved CR **(P<0.005).**Concerning cases that showed double positivity of both genes: 3/18 (16.6%) of them achieved CR while 15/18 (83.3%) didn't achieve CR after induction chemotherapy **(p<0.005).**

**Conclusion:** The present data suggest that MRP&LRP genes expression affects complete remission in Acute leukemia patients. Thus , determination of these genes expression at diagnosis appears likely to provide useful prognostic information for acute leukemia patients.

## Introduction

Drug resistance is a major obstacle in the successful treatment and an important cause of death in acute leukemia. Such resistance may be present before beginning treatment or may develop during chemotherapy. Drug resistance that extends to structurally and functionally unrelated drugs is termed multidrug resistance(MDR) ***(1).***

Clinical resistance to chemotherapy in acute leukemia is often associated with the expression of (membrane) transport-associated multidrug resistance (MDR) proteins ***(2).***

Several molecular biological mechanisms have been identified as being associated with MDR **(3).** P-glycoprotein (PGP), also named P-170, is a product of the multidrug resistance 1 gene(MDR1) and is an ATP-dependent pump capable of expelling drugs out of cancer cells (**4**). Another protein, the multidrug resistance protein (MRP), is structurally similar to PGP and belongs to the same transmembrane transporter superfamily **(5).** The substrate specificity of MRP is similar to but more limited than that of PGP, and its normal physiological role may be detoxification of intracellular oxidants **(6).**

Lung Resistance Protein (LRP), a 110KDa protein was first identified in a PGP-negative MDR lung cancer cell lines and acts as a major vault protein in humans **(7).**

Despite the identification of these proteins, the pathways that result in drug resistance in leukemic cells remain largely uncharacterized. New information regarding drug resistance mechanisms is likely to increase the chances of cure either through development of new drugs or by means of strategies that may modulate or reverse resistance **(8).** While drug resistance gene expression has been studied in acute leukemia **(9 &10),** the value of MRP and LRP gene expression as independent predictors of success of treatment is still controversial. The present study aimed for the detection of expression of the multidrug resistance genes MRP and LRP in acute leukemia and to correlate such expressions with complete remission, clinical and laboratory variables.

## Materials and Methods

### Patients

The study involved 50 patients (30 males and 20 females) diagnosed with denovo acute leukemia between January 2010 and June 2010 recruited from the

National Cancer Institute, Egypt. The median age of the patients was 28 years, with mean 25.3 years (range 2yr-75yr). Diagnosis and classification of acute leukemia was made according to the French-American-British (FAB) criteria and immunophenotype analyses. There were 25 acute myeloid leukemia (AML) and 25 acute lymphoblastic leukemia(ALL) patients. Among the 25 AML patients, two patients had AML M0, 7 M1, 8 M2, 2 M3, 3 M4, 2 M5, 1 M6.

Among the 25 ALL patients, fifteen patients were B-lineage, 6 T-lineage and 4 common ALL.

Ten normal age matched individuals were included as control group

**Complete remission(CR)**

Complete remission was defined as normocellular bone marrow with less than 5% blasts after induction chemotherapy, no Auer rods, and no evidence of extramedullary involvement***(Marry et al;2007)***. Patients who relapsed or died within 28 days after CR were considered as not having achieved a CR.

**Sample collection and Nested RT-PCR assay**

Fifty peripheral blood samples collected in EDTA were obtained at the initial diagnosis. A COR-L23/R cell line was used as a positive control for *LRP* m RNA expression, and the HL60/Adr cell line for *MRP* m RNA expression. RNAase-free water was used as negative control, also ten age matched normal individuals were included as control group. B actin m RNA amplification was used as an internal control.

Mononuclear cells were separated from peripheral blood samples by density gradient centrifugation using Ficoll-Hypaque and preserved at -20ºC till use. Total cellular RNA was extracted from MNCs using a QIAamp RNA blood kit (Qiagen,Germany) . Complementary DNA (cDNA) was synthesized using Revert Aid™ First strand c DNA synthesis kit(Fermentas,K1621). The reaction was performed at 70ºC for 2 min and 42ºC for 60 min. MRP and LRP m RNA amplifications were performed after heating the reaction mixture to 99ºC for 5 min.

The first round of PCR reactions involved 30 cycles of: 1 min denaturing at 94ºC, 1 min annealing at 64ºC and 2 min extension at 72ºC using a thermocycler. The PCR reactions were carried out in a final volume of 25$\mu$l containing 12.5 $\mu$l Dream Tag Green PCR Master Mix(Fermentas,K0171 which contains TaqDNA polymerase in reaction buffer MgCl2 and Dntps), 5/$\mu$l of 5pmol of each primer, 2 $\mu$l c DNA, completed to the final volume with nuclease free water. The first round of PCR was followed by a second round of 20 cycles. Amplification of β-actin m RNA (20 cycle PCR) was performed and the data obtained used to normalize any variation in samples. PCR primer sequences are shown in Table 1. PCR products were electrophoretically separated on 2% agarose gel and visualized using ethidium bromide staining. The sample was considered positive when a clear sharp band was observed at the specific molecular weight;420 bp for MRP, 239 bp for LRP and 166 bp for β actin. The gel was photographed with Polaroid film.

**Statistics**

Data were summarized and presented in the form of percentage, range , mean and median. Descriptive statistics and statistical comparisons were performed using the statistical soft ware program SPSS (version 14). A ***P*** value of < 0.05 indicated a significant difference while a ***p*** value of > 0.05 indicated insignificant difference.

**Table 1: NestedRT-PCR primer sequences for MRP, LRP and β- actin m RNA amplification (1)**

| Target | | Direction | Sequences of primer |
|---|---|---|---|
| MRP | External primers | Sense | 5′-TACACCGTGCTGCTGTTTGTCACT-3′ |
| | | Antisense | 5′-GTCTTGGTCATCGCCATCACA-3′ |
| | Internal primers | Sense | 5′-ACTTCCACATCTGCTTCGTCAGTG-3′ |
| | | Antisense | 5′-ATTCAGCCACAGGAGGTAGAGA-GC-3′ |
| LRP | External primers | Sense | 5′-GTCTTCGGGCCTGAGCTGGTGT-CG-3′ |
| | | Antisense | 5′-CTTGGCCGTCTCTTGGGGGTCC-TT-3′ |
| | Internal primers | Sense | 5′-TTCTGGATTTGGTGGACG-3′ |
| | | Antisense | 5′-ACTTCTCTCCCTTGACCA-3′ |
| β-actin | | Sense | 5′-GTGGGGCGCCCCAGGCACCA -3′ |
| | | Antisense | 5′-GTCCTTAATGTCACGCACGATTTC-3′ |

## Results

**Expression rates of m RNA of MRP and LRP genes:**

By Nested RT-PCR, m RNA of MRP and LRP genes were detected in 28/50 (56%) & 22/50 (44%) respectively, while there was double expression of both genes in 18/50 (36%) of acute leukemia patients.

In terms of leukemia categories, MRP was expressed in 14/25 (56%) of AML patients and 14/25 (56%) of ALL patients; while LRP was expressed in 12/25 (48%) of AML Patients and 10/25 (40%) of ALL patients.

All normal controls didn't express both genes.

Comparison between MRP&LRP positive and negative patients regarding their clinical and laboratory data is presented in table (2).

There was no statistically significant difference between MRP&LRP positive and negative patients as regards their clinical and laboratory data including age, sex , hemoglobin, platelets, hepatosplenomegally, lymphandenopathy and CNS involvement.

As regards Total leucocytic count (TLC) the comparison between positive and negative cases showed high statistically significant difference **(p<0.005).**

**Association of MRP and LRP m RNA expression with the rate of complete remission (CR) after induction chemotherapy:**

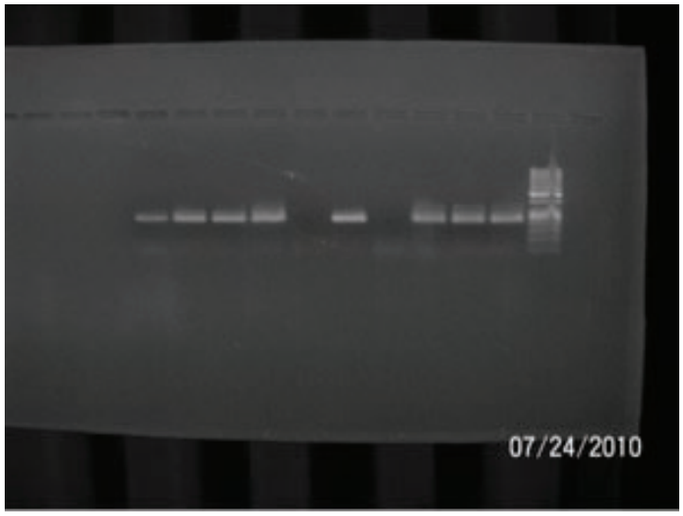
MRP&LRP m RNA expression at initial diagnosis was associated with a lower CR rate after induction chemotherapy, with only 6/28 (21.4%) of MRP positive acute leukemia patients achieving CR, compared to 15/22 (68.1%) of MRP negative acute leukemia patients achieving CR **(P<0.05),** while 3/22 (13.6%) of LRP positive acute leukemia patients achieved CR compared to 18/28 (64.3%) of LRP negative acute leukemia patients achieved CR **(P<0.005).**

Concerning cases that showed double positivity of both genes: 3/18 (16.6%) of them achieved CR while 15/18 (83.3%) didn't achieve CR after induction chemotherapy **(p<0.005).**

**Table 2: Relationship between MRP&LRP genes expression and clinical and laboratory data of patients**

| | MRP | | LRP | |
|---|---|---|---|---|
| | positive | negative | positive | negative |
| **Acute leukemia (n=50)** | 28/50 (56%) | 22/50 (44%) | 22/50 (44%) | 28/50 (56%) |
| **AML (n=25)** | 14/25 (56%) | 11/25 (44%) | 10/25 (40%) | 15/25 (60%) |
| **ALL (n=25)** | 14/25 (56%) | 11/25 (44%) | 12/25 (48%) | 13/25 (52%) |
| **Sex** | | | | |
| **Male(n=24)** | 17/24 (70.8%) | 7/24 (29.2%) | 13/24 (54.2%) | 11/24 (45.8%) |
| **Female(n=26)** | 12/26 (46.2%) | 14/26 (53.8%) | 9/26 (34.6%) | 17/26 (65.4%) |
| **TLCx10³/cm³ (mean&range)** | 90.35* (3-325) | 40.3* (1.35-115) | 92.4* (2.5-325) | 49.6* (1.35-213) |
| **Hemoglobin gm/dl (mean&range)** | 8.1 (4.4-11.6) | 7.6 (2.5-10) | 7.2 (2.3-11.4) | 7.4 (2.5-11.6) |
| **Plateletsx10³/cm³ (mean&range)** | 98.4 (8-413) | 110.6 (6-765) | 128 (8-765) | 96.5 (6-413) |
| **Hepatosplenomegaly** | 10/28 (35.7%) | 8/22 (36.4%) | 10/22 (45.5%) | 12/28 (42.8%) |
| **Lymphadenopathy** | 8/28 (28.5%) | 7/22 (31.8%) | 9/22 (40.9%) | 6/28 (21.4%) |
| **CNS involvement** | 4/28 (14.2%) | 3/22 (13.6%) | 2/22 (9.09%) | 3/28 (10.7%) |
| **Complete Remission** | 6/28* (21.4%) | 15/22* (68.2%) | 3/22* (13.6%) | 18/28* (64.3%) |

***p value<0.05***




***1 2 3 4 5 6 7 8 9 10 S***

**Fig. 1: Nested RT-PCR product of LRP gene (239bp)**
**Lanes 1,2,3,4,6,8,9,10 show positive cases**
**Lanes 5 and 7 show negative cases**
**Lane S: 50-1000 bp ladder size marker**


**Fig. 2: Nested RT-PCR product of MRP gene (420bp) and β actin (166bp)**
**Lanes 1&2 show positive MRP cases**
**Lanes 3,7&8 positive β actin cases**
**Lanes 4,5 &6 negative cases**
**Lane S: 100-1000 bp ladder size marker**

## Discussion

Although the antineoplastic drugs currently available are usually effective for the treatment of various tumors, they may prove to be relatively ineffective in the treatment of some primary or recurrent neoplasia.

The identification of factors that might effectively predict the response of the patient to treatment is a constant challenge in oncology. Cell resistance to drugs is a determinant of the response to chemotherapy and radiotherapy and its detection may be of clinical relevance.

During the last decade, several studies have sought to define the role of expression of transmembrane carriers such as the MRP and LRP genes in the survival from and risk of relapse for acute leukemia **(11).**

One of the mechanisms of drug resistance in cancer cells is associated with altered anticancer drug transport, mediated by members of the ABC (ATP-binding cassette) superfamily of transport proteins **(12)** such as MDR1 and MRP**(13).**

MRP1 is an ABC membrane transport protein implicated in clinical drug resistance and capable of actively decreasing the intracellular drug concentration in functional in vitro assays **(14).** Vesicular transport experiments have shown that the preferred substrates of MRP1 are drugs conjugated to glutathione,glucoronate,or sulfate **(15).** Transfection studies established that MRP1 overexpression confers resistance to a wide variety of anticancer drugs **(14).**

In addition to an overall reduction of intracellular drug concentration, associated with overexpression of ABC transport proteins, a redistribution of the drug from the nucleus to the cytoplasm has also been implicated in MDR of cancer cells **(16).** It has been reported that LRP, as an integral part of the vault complex, is involved in the intracellular distribution of chemotherapeutic agents **(17).** Clinical data indicate that this LRP protein is often expressed in human malignancies and that its expression may be associated with poor response to chemotherapy in ovarian carcinoma and AML **(17).**

Since the expression of MDR genes were found to be of growing importance in the treatment and outcome of leukemia patients ,we investigated by Nested

RT-PCR the m RNA of two genes named MRP&LRP genes. Fifty patients (30 males and 20 females) diagnosed with denovo acute leukemia between January 2009 and December 2009 recruited from the National Cancer Institute, Egypt were included in this study. The median age of the patients was 28 years, with mean 25.3 years (range 2yr-75yr). Diagnosis and classification of acute leukemia was made according to the French-American-British (FAB) criteria and immunophenotype analyses. There were 25 acute myeloid leukemia (AML) and 25 acute lymphoblastic leukemia(ALL) patients. Among the 25 AML patients, two patients had AML M0, 7 M1, 8 M2, 2 M3, 3 M4, 2 M5, 1 M6.

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Ten normal age matched individuals were included in our study as control group.

m RNA of MRP and LRP genes were detected in 28/50 (56%) & 22/50 (44%) respectively, while there was double expression of both genes in 18/50 (36%) of acute leukemia patients.

In terms of leukemia categories, MRP was expressed in 14/25 (56%) of AML patients and 14/25 (56%) of ALL patients; while LRP was expressed in 12/25 (48%) of AML Patients and 10/25 (40%) of ALL patients.

All normal controls didn't express both genes.

There was no statistically significant difference between MRP&LRP positive and negative patients as regards their clinical and laboratory data including age, sex , hemoglobin, platelets, hepatosplenomegally, lymphandenopathy and CNS involvement.

**Mary M. et al (2007)(2)** showed that the m RNA expression of resistance genes were not significantly associated with the age of patients.

As regards Total leucocytic count (TLC) the comparison between positive and negative cases showed high statistically significant difference ($p<0.005$) this was in accordance with **Mary M.,et al (2007)(2),** they stated in their study that there was a positive correlation between the expression of MRP&LRP and high white blood cell count($p<0.001$) in AML patients.

MRP&LRP m RNA expression at initial diagnosis was associated with a lower CR rate after induction chemotherapy, with only 6/28 (21.4%) of MRP positive acute leukemia patients achieving CR, compared to 15/22 (68.1%) of MRP negative acute leukemia patients achieving CR **($P<0.05$),** while 3/22 (13.6%) of LRP positive acute leukemia patients achieved CR compared to 18/28 (64.3%) of LRP negative acute leukemia patients achieved CR **($P<0.005$).**

Concerning cases that showed double positivity of both genes: 3/18 (16.6%) of them achieved CR while 15/18 (83.3%) didn't achieve CR after induction chemotherapy **($p<0.005$).**

In agreement with our results, **Huh H.J. et al (2006)(1)** stated in their study that LRP m RNA expression at initial diagnosis was associated with a lower CR rate after induction chemotherapy, with only 55.2%(16/29) of LRP-positive acute leukemia patients achieving CR, compared to 82.9% (29/35) of LRP-negative patients achieving CR ($p=0.02$). Similarly, for AML patients, 47.1%(8/17) of LRP-positives achieved CR, while 82.4%(14/17) of LRP-negatives achieved CR ($P=0.03$). They also stated that MRP gene m RNA expression appeared to have statistically no significant effect on patient outcome following induction chemotherapy. However MRP positive patients showed the tendency of lower CR than MRP negative cases.

**Huh H.J. et al (2006)(1)** also stated in their study that double positivity of MRP& LRP had an effect on CR rate; hey stated that while 57.1%(16/28) of acute leukemia patients expressing both genes m RNA achieved CR,80.6%(29/36) of those not expressing both genes achieved CR($P=0.04$).

On the other hand **Mary M., et al (2007)(2)** stated in their study that MRP and LRP m RNA were not associated with CR rate or survival endpoints in patients with AML.

**Valera E.T. et al (2004)(11)** also stated that the expression of the MRP gene upon diagnosis was not related to a worsening of event free survival in childhood ALL, but they observed that increased LRP upon diagnosis was strongly related to worsened event free survival. Patients with positive LRP upon diagnosis presented a cumulative survival rate of 25% over 48 month, in comparison with 83.5% cumulative rate of LRP-negative patients($p=0.005$).

**Sauerbrey etal (2002)(18)** also did not find a correlation between MRP expression upon diagnosis and a worsened survival of children with a diagnosis of ALL, but they demonstrated that children with a diagnosis of ALL and high LRP expression, as assessed by semiquantitative RT-PCR, showed a lower tendency to remain in first clinical remission,although this finding did not reach statistical significance.

**Plasschaert et al (2003)(19)** in a study on the determination of MRP by semiquantitative RT-PCR and flowcytometry, showed that increased MRP expression upon diagnosis had no impact on the event-free survival of children or adults.

**Kakihara et al (1999)(9)** also did not detect an adverse impact from high MRP expression upon diagnosis, on the survival of children with ALL, and they also found no correlation between the expression of LRP gene, as determined by RT-PCR & variables such as age and leucocytic count upon diagnosis, nor did they observe worsened survival in patients with high LRP upon diagnosis.

**den Boer et al (1998)(20)** did not detect an adverse impact from LRP expression upon diagnosis, but only observed an increase in LRP expression in multiple relapses.

In conclusion, the present study demonstrates that outcomes in acute leukemia patients can vary depending upon expression of MRP and LRP m RNA. Simultaneous expression of MRP and LRP m RNA correlated with a low CR rate. These results suggest that analysis of MDR gene expression at diagnosis of acute leukemia may provide useful prognostic information. Such data is also likely to assist in determining the mechanisms underlying drug resistance.

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