RESEARCH ARTICLE

High Expression of Lung Resistance Protein mRNA at Diagnosis Predicts Poor Early Response to Induction Chemotherapy in Childhood Acute Lymphoblastic Leukemia

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Abstract

Background: Treatment failure in leukemia is due to either pharmacokinetic resistance or cell resistance to drugs. Materials and Methods: Gene expression of multiple drug resistance protein (MDR-1), multidrug resistance-related protein (MRP) and low resistance protein (LRP) was assessed in 45 pediatric ALL cases and 7 healthy controls by real time PCR. The expression was scored as negative, weak, moderate and strong. Results: The male female ratio of cases was 2.75:1 and the mean age was 5.2 years. Some 26/45 (58%) were in standard risk, 17/45(38%) intermediate and 2/45 (4%) in high risk categorie, 42/45 (93%) being B-ALL and recurrent translocations being noted in 5/45 (11.0%). Rapid early response (RER) at day 14 was seen in 37/45 (82.3%) and slow early response (SER) in 8/45 (17.7%) cases. Positive expression of MDR-1, LRP and MRP was noted in 14/45 (31%), 15/45 (33%) and 27/45 (60%) cases and strong expression in 3/14 (21%), 11/27 (40.7%) and 8/15 (53.3%) cases respectively. Dual or more gene positivity was noted in 17/45 (38%) cases. 46.5 % (7/15) of LRP positive cases at day 14 were in RER as compared to 100% (30/30) of LRP negative cases (p<0.05). All 8 (100%) LRP positive cases in SER had strong LRP expression (p=<0.05). Moreover, only 53.3% of LRP positive cases were in haematological remission at day 30 as compared to 100% of LRP negative cases (p=<0.05). Conclusions: Our study indicated that increased LRP expression at diagnosis in pediatric ALL predicts poor response to early treatment and hence can be used as a prognostic marker. However, larger prospective studies with longer follow up are needed, to understand the clinical relevance of drug resistance proteins.

Keywords: ALL - chemotherapy - drug resistance proteins - pediatric - response

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Introduction

Acute lymphoblastic leukemia (ALL) is the commonest malignancy in children, comprising about 30-35% of all childhood cancers (Kaatsch et al., 2010). With advancements in chemotherapy regimen and sensitive techniques to monitor disease status, 80-85% of children and adolescents with ALL, get cured completely (Pui et al., 2012; Nigro, 2013). However, the overall survival rates for pediatric ALL are still lower in many developing countries as compared to west (Naureen et al., 2013; Panya et al., 2015) and approximately 25% of pediatric ALL cases develop disease recurrence despite receiving standard chemotherapy regimens. Furthermore, only 20-30% of children with leukemic relapse have a long-lasting second remission with the chance of cure with second-line treatment (Rivera et al., 1991; Gaynon et al., 1993; Bhadri et al., 2012).

Treatment failure can be explained in part by pharmacokinetic mechanisms that reduce the time length or effective level of leukemic blast exposure to the drug, also known as pharmacokinetic resistance. It can also be partially explained by cell resistance to drugs (Pieters et al., 1997). Cell resistance to antineoplastic drugs is seen as one of the most significant barriers against effective treatment of malignant tumors in general. The description of the multiple drug resistance protein (MDR-1), also called p-glycoprotein, seemed promising for the understanding of the mechanisms of anti-neoplastic treatment failure. Subsequently, other drug resistance genes were described, among them genes related to resistance (multidrug resistance-related protein, MRP) and genes for the lung resistance protein (LRP) were found to be of clinical and prognostic significance. A study by (Wanida et al., 2015), found polymorphisms in MDR1 gene to be associated with high risk pediatric ALL but others like (Madara et al., 2014) failed to find any relation between MDR1 polymorphism and pediatric ALL risk. Similarly, the relationships between MDR-1, MRP and LRP expression, resistance to treatment and survival

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among children with acute lymphoblastic leukemia are still unclear from various studies published (Boer et al., 1998; Norgaard et al., 2000).

Studies on above subject from our subcontinent are very limited. With an increasing trend towards individualized treatment regimens based on patient's prognostic genotyping data, studies on expression of drug resistance proteins and their relation with treatment outcome, assumes significance. The present study was done with an aim to note expression of MDR-1, MRP and LRP genes at diagnosis in pediatric ALL cases and to study their relation with early response to therapy and other clinico-laboratory parameters.

Materials and Methods

A prospective study in which 45 pediatric ALL cases admitted for treatment in Hematology Oncology unit of our hospital over a period of one year (July 2-13-June 2014) were enrolled. The cases were confirmed as ALL on bone marrow examination and flow cytometry based immunophenotyping using standard panel of monoclonal antibodies. The study was duly approved by the ethics committee of our institute.

Messenger RNA Expression of MDR, MRP and LRP Genes

Five milliliter of EDTA blood was collected from each subject and 7 healthy control subjects under the guidelines of Institutional Ethics Committee and after obtaining the signed consent from the parent or their guardian. Briefly, blood sample was over layered slowly from an angle onto the density gradient solution (Ficoll-Hypaque, Sigma-Alderich, St. Louis, MO, USA) in a 15 ml tube and was centrifuged for 30 min at 1800rpm. A white buffy coat layer of PBMCs (peripheral blood mononuclear cells) was aspirated out from the tube using pasture pipette in a 1.5 ml centrifuge tube. Cells were pelleted by centrifuging at 2500rpm for 5 min. Total RNA from the PBMCs was extracted using the Qiamp RNA blood mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. After measuring the concentration by taking the OD on Nanodrop 2000 (Thermo Fisher, Waltham, MA, USA), 1ug of total RNA was reverse transcribed to cDNA in 20uL reaction volume using the Fermantas cDNA Reverse Transcription Kit with RNase Inhibitor. The reaction was performed at 25°C for 5 min and 37°C for 120 min followed by 80°C for 5 min.

For Real-Time PCR, cDNA equivalent to 10ng of total RNA was used for each reaction of 10ul containing 1.0ul (10picomol of each) primers, 2ul (10ng) cDNA (diluted ten times), 5ul of 2X SYBR Green Mix (Applied Biosystems, Foster City, CA, SUA) and made up to the final volume with sterile distilled water. Reactions were performed in Step One Plus Real-Time PCR system (Applied Biosystems, Foster City, CA). Primers for MDR1, MRP1 and LRP used here were adopted from the published work of (Valera et al., 2004) and GAPDH primers were self-designed using free online software Primer 3 (v.0.4.0). All the primers used were synthesized from Eurofins India (Bangalore, India) having Tm between 61.5°C-62.2°C. The sequence detail is given in the Table 1 below.

Real-Time (RQ) PCR amplifications for MDR1, MRP1 and LRP mRNA were performed after heating the reaction mixture to 95°Cfor 10 min followed by 40 cycles of: 30Sec denaturation at 95°C and 1min annealing, extension and simultaneous acquisition of signals of SYBR Green at 60°C. Amplification of GAPDH mRNA (40 cycle RQ-PCR) was performed and the data obtained was used to normalize as endogenous control for calculating relative expression of target genes and to know if there were any variations between samples (e.g. RNA quality obtained from the kit). Also melting curve was obtained after each run to ensure the single specific signal peak from the amplification reaction (Figure 1).

Analysis of Real-Time PCR data

After ensuring the optimization of amplification reactions the mean Ct Value for each target gene (MDR1, MRP1 and LRP) from each sample was used for measuring the difference in Ct values between samples and controls

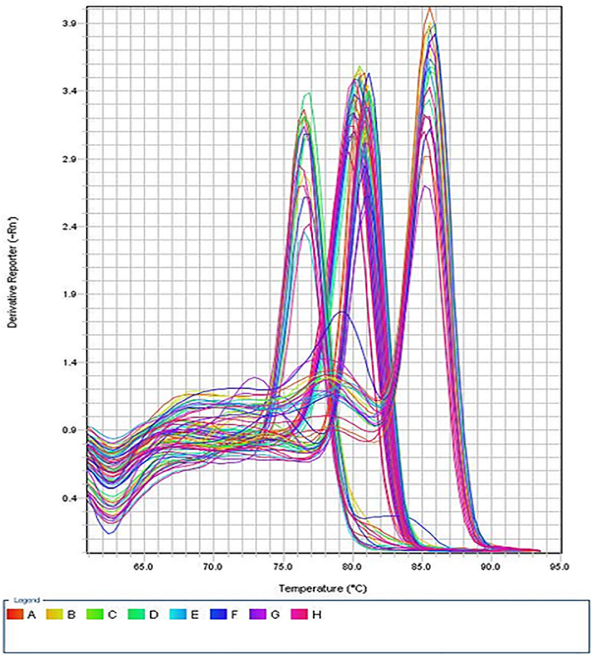

Figure 1. Tim from Melting Curve for PCR Product for all three MDR Genes and GAPDH Melt Curve

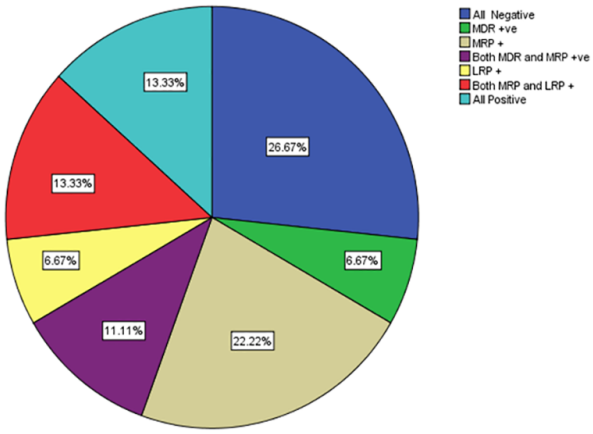

Figure 2. Pie-chart Highlighting Target Gene Expression in ALL Cases

while keeping the GAPDH as endogenous control. The relative expression was quantified by calculating the fold change in expression or Relative Quotient (RQ) using the Delta-Delta-Ct method. The expression of target genes in relation to internal control gene (GAPDH) was scored as negative (<0 fold expression;0) weak (0-2 fold higher;1+), moderate (2.1-4 fold higher; 2+) and strong (>4 fold higher; 3+).

The correlation of the MDR-1, MRP and LRP genes with age upon diagnosis, leukocyte count, organomegaly, immunophenotypic classification, presence of recurrent transcripts and early response to chemotherapy (Day 14-check bone marrow) and Day 30 post induction response was also assessed. The response criteria to chemotherapy were defined as follows:-

1. Day 14 bone marrow for percentage of blasts. The response was classified as rapid early response (RER) and slow early response. RER- M1 response (<5 % blasts) and SER - M2 (5-20% blasts) and M3 (> 21% blasts) response.

2. Day 30 post induction response based on peripheral blood criteria as follows:F

i) Complete hematological remission (CHR): Hb > 100 gm/L, TLC 4 -12×10^9/L, platelets 1.5-4.5×10^9/L and no blasts in peripheral blood. *ii*) Partial hematological remission (PHR): Either of the above hematological parameters not in normal range but no blasts in peripheral blood. *iii*) No hematological remission (NHR): Presence of blasts in peripheral blood irrespective of range of hematological parameters.

The ALL cases were treated as per UK MRC 2003 modified protocol and classified into standard, intermediate and high risk groups as per NCI criteria based

Table 1. Sequence of Internal Control and Target Gene Primers

Gene Symbols	Primers (5'-3')	PCR Product Size (bp)	Tm (°C) from Melting Curve of PCR Product
GAPDH	F: ACATCGCTCAGACACCATG R: TGTAGTTGAGGTCAATGAAGGG	143	80.47
MDR	F: CCCATCATTGCAATAGCAGG R: GTTCAAACTTCTGCTCCTGA	157	80.83
MRP	F:TGGGACTGGAATGTCACG R:AGGAATATGCCCCGACTTC	242	85.5
LRP	F: GTCTTCGGGCCTGAGCTGGTGTCG R: CTTGGCCGTCTCTTGGGGGTCCTT	216	76.48

Table 2. Relative Quantification expression of MDR-1, MRP and LRP genes

Leukemia	Number of cases	MDR-1 (+)			MRP (+)			LRP (+)		
		1 (+)	2 (+)	3 (+)	1 (+)	2 (+)	3 (+)	1 (+)	2 (+)	3 (+)
Acute Lymphoblastic Leukemia (ALL)	45	14 (31%)			27 (60%)			15 (33.3%)		
		3(6.7)	8(17.8)	3(6.7)	10(22)	6(13)	11(25)	5(11)	2(4.3)	8 (18)

Table 3. Correlation of Demographic, Clinico-haematological and Chemotherapy Response Variables with MDR-1, MRP and LRP Gene Expression

S. No.	Variables	MDR-1 (%)		MRP (%)		LRP (%)	
		(-)	(+)	(-)	(+)	(-)	(+)
1	Age- <1 or > 10 yrs	4 (12.9)	1 (7.1)	2 (11.1)	3 (11.1)	4 (13.3)	1 (6.7)
	1-10 yrs	27 (87.1)	13 (92.9)	18 (88.9)	22 (88.9)	26 (86.7)	14 (93.3)
		P=0.569	P=1.0	P=0.502			
2	Sex - Male	23 (74.2)	9 (64.3)	12 (66.7)	20 (74.1)	19 (63.3)	13 (86.7)
	Female	8 (25.8)	5 (35.7)	6 (33.3)	7 (25.9)	11 (36.7)	2 (13.3)
		P=0.497	P=0.591	P=0.104			
3	NCI Group-Standard	19 (61.3)	7 (50)	11 (61.1)	15 (55.6)	17 (56.7)	9 (60)
	Intermediate	9 (29) 6 (42.9)	5 (27.8)	10 (37)	11 (36.7)	4 (26.7)	
	High	3 (9.7)	1 (7.1)	2 (11.1)	2 (7.4)	2 (6.7)	2 (13.3)
		P=0.659	P= 0.778	P=0.664			
4	Immunophenotype B	29 (93.5)	13 (92.9)	16 (88.9)	26 (96.3)	27 (90)	15 (100)
	T	2 (6.5)	1 (7.1)	2 (11.1)	1 (3.7)	3 (10) 0 (0)	
		P=0.931	P= 0.329	P=0.205			
5	Translocations - (P)	3 (9.7)	2 (14.3)	2 (11.1)	3 (11.1)	2 (6.7)	3 (20)
	(N)	28 (90.3)	12 (85.7)	16 (88.9)	24 (88.9)	28 (93.3)	12 (80)
		P=0.649	P=1.0	P=0.180			
6	Day 14 status- RER	25 (80.6)	12 (85.7)	15 (83.3)	22 (81.5)	30 (100)	7 (46.7)
	SER	6 (19.4)	2 (14.3)	3 (16.7)	5 (18.5)	0 (0)	8 (53.3)
		P=0.681	P=0.874	P=0.000			
7	Day 30 status- CHR	23 (74.2)	12 (85.7)	13 (72.2)	22 (81.5)	28 (93.3)	7 (46.7)
	NHR	5 (16.1)	2 (14.3)	3 (16.7)	4 (14.8)	0 (0) 7	(46.7)
		P=0.463	P=0.597	P=0.000			

Table 4. MDR Gene Expression Studies in Childhood ALL

Study	Methodology	Number of cases	Conclusion
El-Sharnouby JA et al (2010)	Real Time PCR	34	LRP and MRP expression associated with lower CR rates and poorer 2yr outcome; increased expression in relapse cases too
Fedasenka UU et al (2008)	Real Time PCR	19	No relation between MDR gene expression and MRD after induction
Kourti M et al (2007)	Semi quantitative RT-PCR	49	High MDR-1 associated with worse prognosis; High MDR-1 also noted at relapse
Zhang JB et al (2005)	Semi quantitative RT-PCR	38	Increased LRP and MRP expression at diagnosis a ssociated with lower CR rates after induction therapy
Sauerbrey A et al (2002)	Semi quantitative RT-PCR	86	No significant association of LRP and MRP expression with outcome
Kakihara T et al (1999)	Semi quantitative RT-PCR	40	No association of MDR-1, MRP and LRP gene expressions with biological risk factors
Volm M et al (1997)	IHC (Immunohistochemistry)	25	Higher event free survival (EFS) in LRP negative cases
ET Valera at al (2004)	Semi quantitative RT-PCR	30	Increased LRP expression associated with worsened EFS and risk of death/ relapse also high in high LRP positive cases
ML Den Boer et al (1998)	Flow cytometry	141	LRP might contribute to drug resistance but only in specific subset of children with leukemia

on age and TLC at presentation. Children were followed up till the completion of study to look for relapse/death.

Statistical analysis

The statistical calculation and tests were performed using the SPSS 21.0 software. The normality of the data was tested using the Shapiro-Wilk normality test. The correlation of MDR-1, MRP and LRP genes with demographic, clinical and other haematological parameters was performed using the Chi-square or Fischer exact test. The level of significance was taken at $p<0.05$ for all analyses.

Results

Forty five peripheral blood samples from children with ALL at diagnosis were collected and processed to note expression of MDR-1, MRP and LRP genes.

There were 32 (71%) male cases and 13 (29%) female with a M: F-2.45:1. The mean age was 5.2 years with 40 (89%) with age between 1-10 years and only 5 (11%) with age <1 or > 10 years. 41 (91%) cases had hepatomegaly, 37 (82%) splenomegaly and 35 (78%) generalised lymphadenopathy at presentation. Based on TLC and age, 26/45 (58%) were in standard risk, 15/45(33%) intermediate and 4/45 (9%) in high risk category. None of the cases had CSF positivity at diagnosis. Only 3/45 (7%) were T-ALL and rest (93%) were B-ALL. Recurrent translocations by RT-PCR were noted in 5/45 (11.0%). Day 14 check marrow status was rapid early response (RER) in 37/45 (82%) and slow early response (SER) in 8/45 (18%) cases. Complete haematological remission (CHR) at day 30 was noted in 35 (78%) cases and 7 (16%) were not in haematological remission. Data in 3 cases for day 30 was not available as they left treatment after day 14. The mean duration of follow up available till completion of study was 4 months. There were a total of 7 deaths, all post induction and sepsis related.

Expression levels of MDR genes

Positive expression of MDR-1 was noted in 14/45 (31%), LRP in 15/45 (33%) and MRP in 27/45 (60%) cases. The MRP gene was expressed in more number of cases than other genes but the rate of LRP expression (strong/3+; > 4 fold) was higher than MRP or MDR-1 genes (Table 2).

Association of MDR-1, MRP and LRP expression with demographic, clinico-haematological variables and early response to chemotherapy

Age, sex, organomegaly, immunophenotype or presence of recurrent translocations had no correlation with MDR-1, MRP and LRP expression at diagnosis (Table 3). However, LRP mRNA expression at diagnosis had significant correlation with poor response to early induction chemotherapy. None of the LRP negative cases at diagnosis had SER at day 14 as compared to 53.3% of LRP positive cases (p=0.000). In addition, all 8 (53.3%) cases with SER at day 14 had strong/> 4 fold (3+) LRP expression at diagnosis (p=<0.05). 7/8 of these cases with SER were also not in remission at day 30 post induction as compared to all LRP negative cases that were in CHR at day 30 (p=0.000). Only one case with disease relapse till last follow up was noted and it also had 4 fold high LRP expression.

Cases expressing two or more target genes

Positive expression of two or more genes was noted in 17/45 (37.7%) cases (Figure 2). Double positivity for MDR-1 and MRP alone was seen in 5 (11.1%) cases and MRP and LRP in 6 (13.3%) cases. None of the cases had MDR-1 and LRP positivity alone. Positivity for all three genes was seen in 6 (13.3%) cases. Two or more gene positivity did not have any correlation with clinical, demographic variables or day 14 and day 30 response to chemotherapy.

33/45 (73.3%) cases were alive till last follow up available. 7/45 (15.5%) cases died and all deaths were post induction due to development of febrile neutropenia and sepsis. 5/45 (11.1%) cases left treatment against medical advice at different time points during the study period. Only one case (2.2%) had disease relapse during

consolidation phase.

Discussion

Multidrug resistant (MDR) genes like MDR-1, MRP and LRP are known to confer chemotherapeutic drug resistance by efflux of drugs from leukemic cell cytoplasm. However, many studies regarding correlation between their degree of expression and induction of early clinical remission, prediction of relapse or impact on overall survival or outcome in pediatric ALL have yielded conflicting results (Table 4). The studies have predominantly employed the flow cytometric technique using monoclonal antibodies against drug resistance proteins or semi quantitatively assessed expression levels of these genes using RT-PCR method. Only few studies have used the more sensitive real time PCR (Fedasenka et al., 2008; Mahjoubi et al., 2012) based analysis or nested PCR (Huh et al., 2006) based methods.

In the present study, the MDR-1, MRP and LRP gene expressions at diagnosis, were studied using real time PCR assay, in pediatric ALL cases. The study shows a high expression of MRP (60%) at diagnosis while LRP (33%) and MDR-1 (31%) expression was comparable to other studies. (Valera et al., 2004) found MDR-1, MRP and LRP expression in 40%, 16.6% and 33% cases respectively at diagnosis in childhood ALL. (El-Sharnouby et al., 2010) found LRP and MRP to be expressed in 41.2% and 35.3% cases respectively. (Kourti et al., 2007) found MDR-1 expression in 36.7% cases and (Volm et al., 1997) noted LRP expression in 47% cases at diagnosis in pediatric ALL. Positive expression of two or more genes was noted in 37.7% of our cases, while (Valera et al., 2004) noted it in 26.6% cases. However, no statistically significant correlations were found between expressions of these genes.

Age, sex, organomegaly/lymphadenopathy at presentation, immunophenotype, NCI risk group based on age and TLC and presence of recurrent translocations did not have any significant association with any of the three MDR genes. Study by (Boer et al., 1998) showed lower MDR-1/P-gp expression in T-ALL as compared to B-ALL, while (Valera et al., 2004) and (Ogretmen et al., 2000) showed correlation of increased MRP and LRP expression with CALLA expression or pre-B ALL immunophenotype. However, we did not found any association between immunological subgroups and MDR gene expressions because of very few number of T-ALL cases in our study. Out of 45, only 3 (7%) cases were of T-ALL and two of these were negative for expression of all three MDR genes.

In our study, LRP expression at diagnosis was found to be significantly associated with early response to induction chemotherapy. LRP mRNA expression at diagnosis was associated with a lower RER at day 14 of induction chemotherapy, with only 7/15 (46.7%) of LRP positive cases achieving RER as compared to 30/30 (100%) LRP negative cases (p=0.000). In addition, all 8 (100%) LRP positive cases in SER at day 14 had strong LRP (>4 fold) expression (p=<0.05). Day 30 post induction chemotherapy response was also poor in LRP positive cases with 46.7% cases not being in haematological remission (NHR) as compared to 100% of LRP negative cases being in complete remission (CHR) at day 30 (p=0.000). Only one case in our study had disease relapse and that case too had 4 fold higher LRP expression at diagnosis and was in NHR at day 30. The remission free interval in this case was 2 months. Many studies (Volm et al., 1997; Boer et al., 1998; Valera et al., 2004; Zhang et al., 2005; Huh et al., 2006; El-Sharnouby et al., 2010) have noted a significant association with increased LRP expression at diagnosis and or relapse to be associated with lower complete remission rates and poorer event free survival and overall outcome (Table 4). Study by (Mahjoubi et al., 2012) found increased MDR1 expression to be associated with relapse in pediatric ALL cases. However few other studies (Boer et al., 1998; Sauerbrey et al., 2002; Fedasenka et al., 2008) have not noted any relation of MDR gene expressions with outcome. Expression of two or more genes was not significantly associated with any biological or treatment response parameter in our study.

The present study highlights that LRP gene expressions at diagnosis is significantly associated with early response and complete remission state post induction in pediatric ALL. However, MDR gene expressions have no correlation with underlying biological risk factors. There is also need for larger prospective studies especially related to study of LRP expression at disease relapse and correlation of its expression with long term survival and outcome.

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